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Call et al.

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(54) **MICROCIN AND USES THEREOF**

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(73) Assignee: **Washington State University**, Pullman, WA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **15/992,874**

(22) Filed: **May 30, 2018**

(65) **Prior Publication Data**

US 2018/0264078 A1 Sep. 20, 2018

Related U.S. Application Data

(60) Division of application No. 15/268,666, filed on Sep. 19, 2016, now Pat. No. 10,022,423, which is a continuation-in-part of application No. 14/407,975, filed as application No. PCT/US2013/045937 on Jun. 14, 2013, now Pat. No. 9,492,500.

(60) Provisional application No. 61/660,616, filed on Jun. 15, 2012.

(51) **Int. Cl.**

A61K 39/02	(2006.01)
A61K 38/16	(2006.01)
A61K 35/74	(2015.01)
A01N 63/02	(2006.01)
A01N 63/00	(2006.01)
A01N 37/46	(2006.01)

(52) **U.S. Cl.**

CPC ***A61K 38/164*** (2013.01); ***A01N 37/46*** (2013.01); ***A01N 63/00*** (2013.01); ***A01N 63/02*** (2013.01); ***A61K 35/74*** (2013.01)

(58) **Field of Classification Search**

CPC A61K 38/164

See application file for complete search history.

(56) **References Cited**

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Primary Examiner — Albert M Navarro

(74) *Attorney, Agent, or Firm* — W&C IP

(57) **ABSTRACT**

Microcin MccPDI and bacteria harboring the mcpM gene which encodes MccPDI limit growth of and/or kill pathogenic bacteria such as pathogenic *Escherichia coli* (*E. coli*) and/or *Shigella* bacteria via proximity-dependent inhibition (PDI).

4 Claims, 17 Drawing Sheets

Specification includes a Sequence Listing.

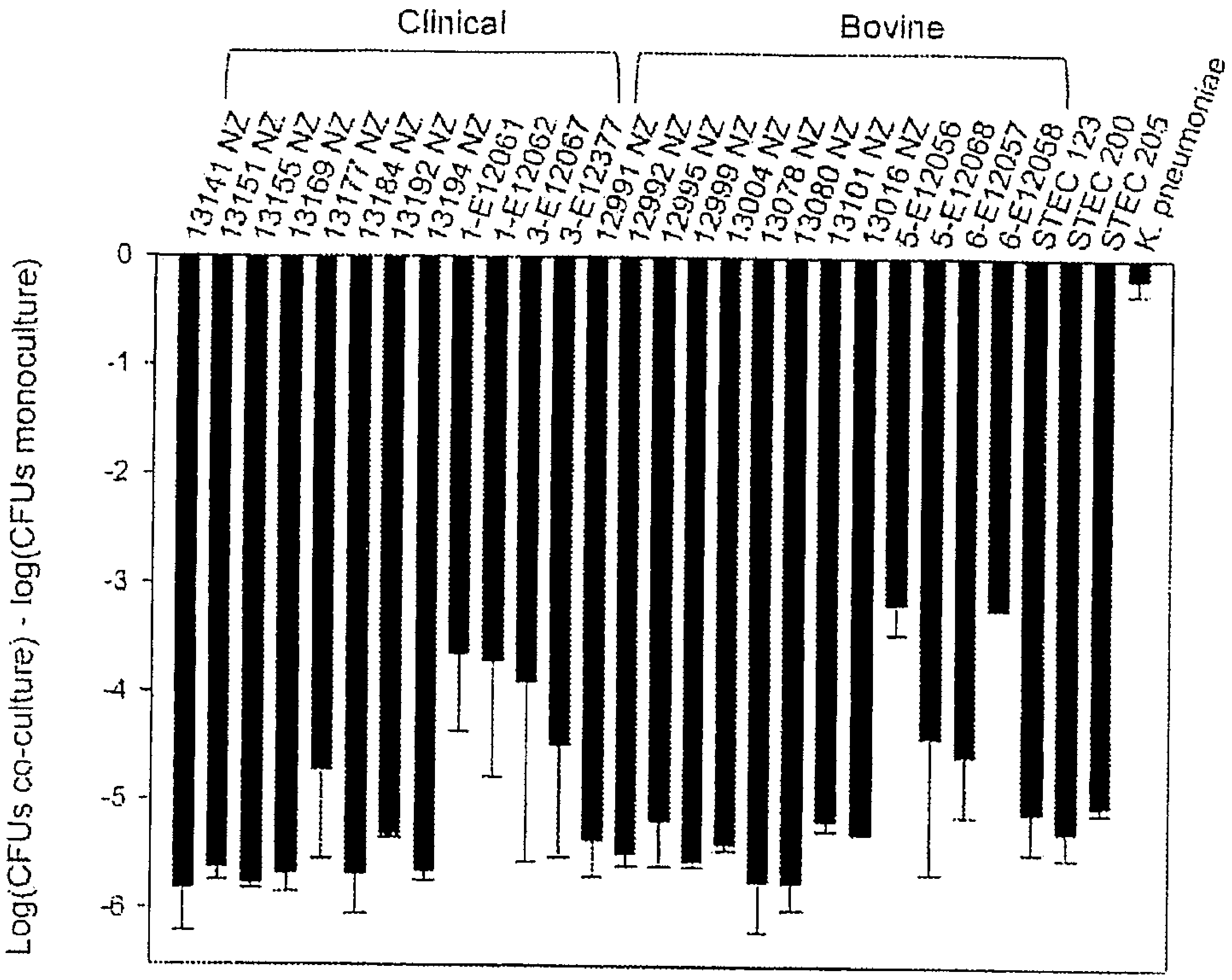


Figure 1

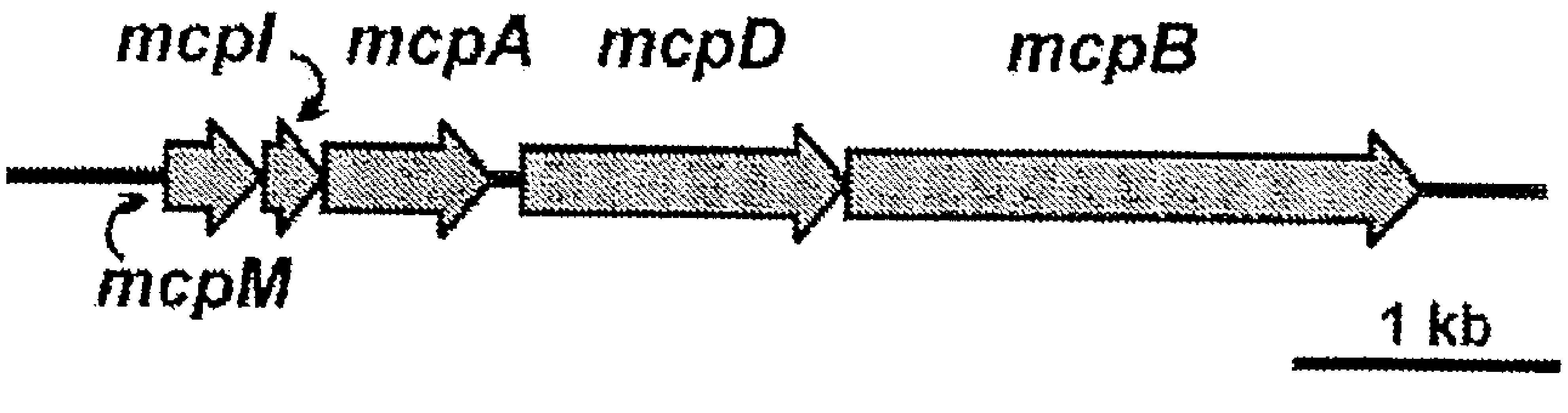


Figure 2

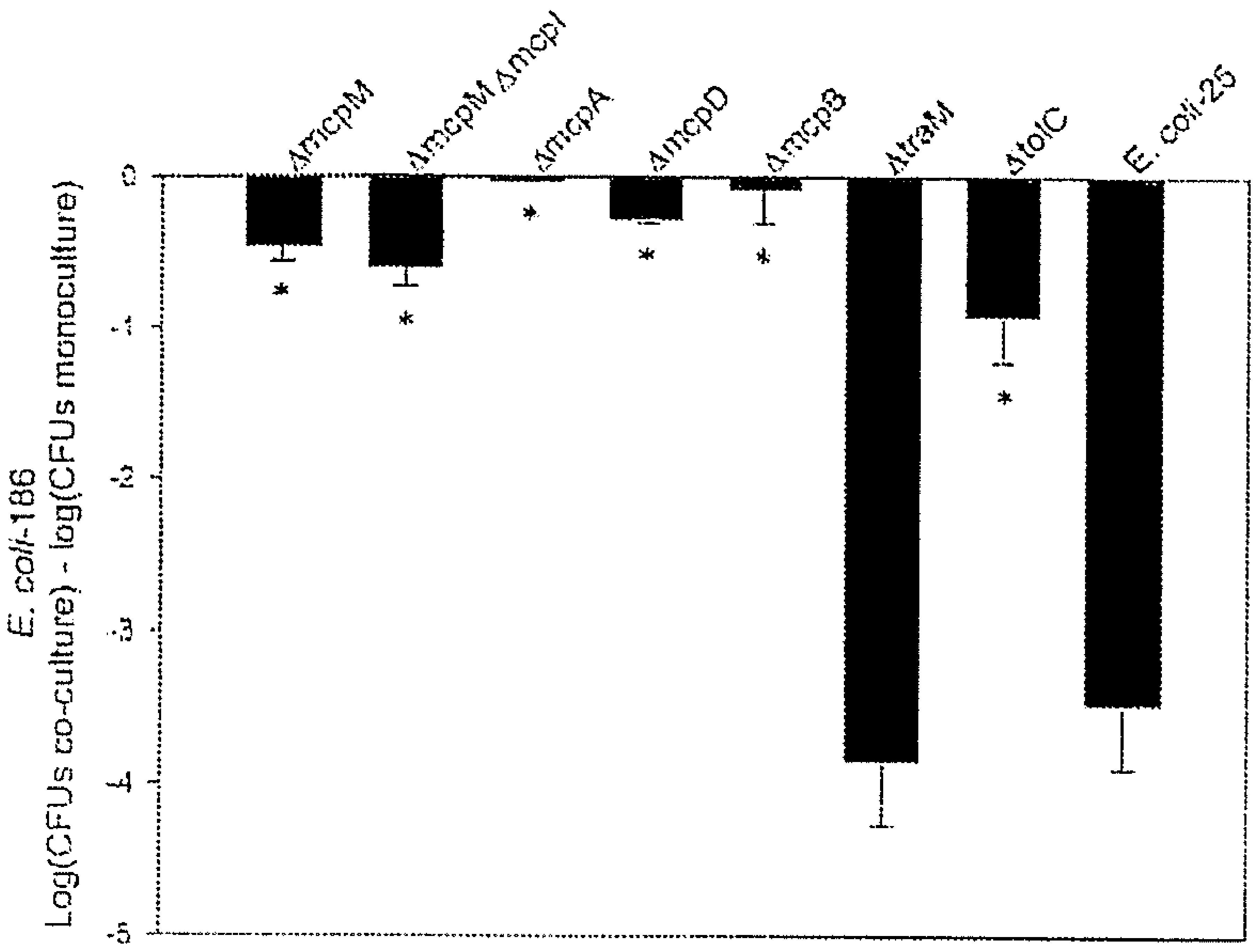

ΔmcpM
ΔmcpMΔmcpI
ΔmcpA
ΔmcpD
ΔmcpB
ΔtraM
ΔtolC
E. coli-25
E. coli-186
Log(CFUs co-culture) - log(CFUs monoculture)
0
-1
-2
-3
-4
-5
*
*
*
*
*
*

Figure 3A

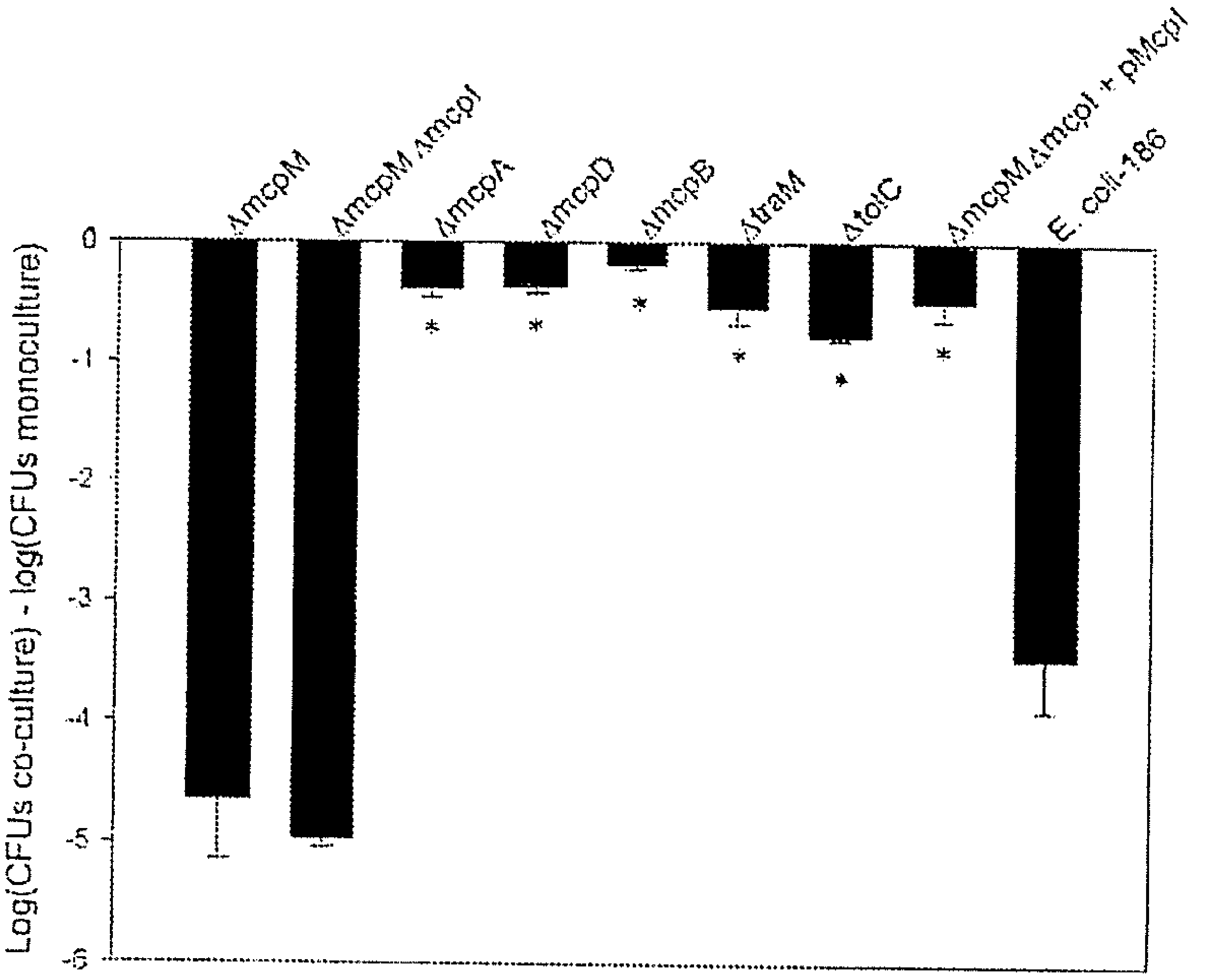

ΔmcpM
ΔmcpMΔmcpI
ΔmcpA
ΔmcpD
ΔmcpB
ΔtraM
ΔtolC
ΔmcpMΔmcpI + pMcpI
E. coli-186
Log(CFUs co-culture) - log(CFUs monoculture)
0
-1
-2
-3
-4
-5
-6
*
*
*
*
*
*

Figure 3B

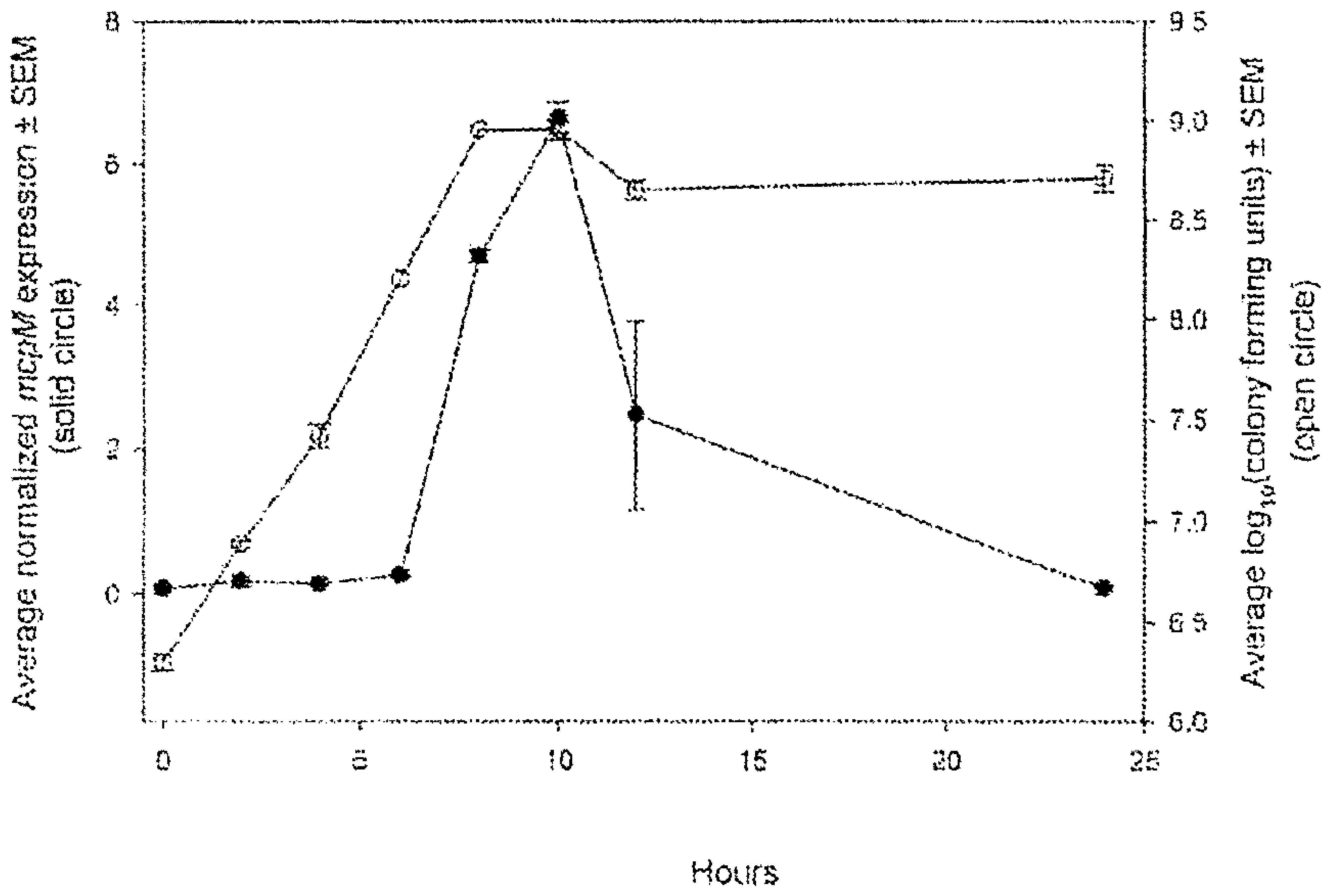

Average normalized mcpM expression ± SEM
(solid circle)
8
6
4
2
0
Average $\log_{10}$(colony forming units) ± SEM
(open circle)
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
0
5
10
15
20
25
Hours

Figure 4

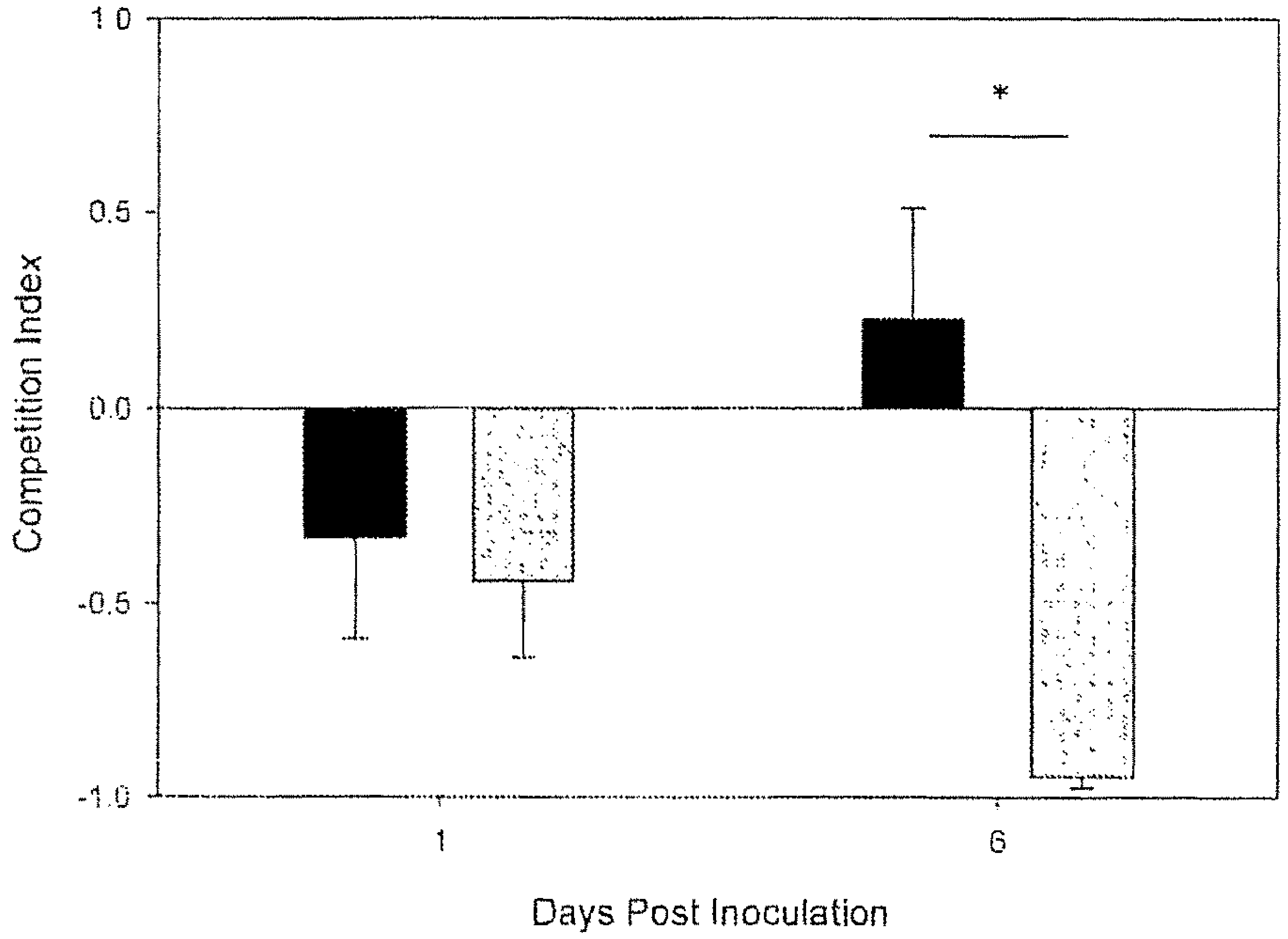


Figure 5

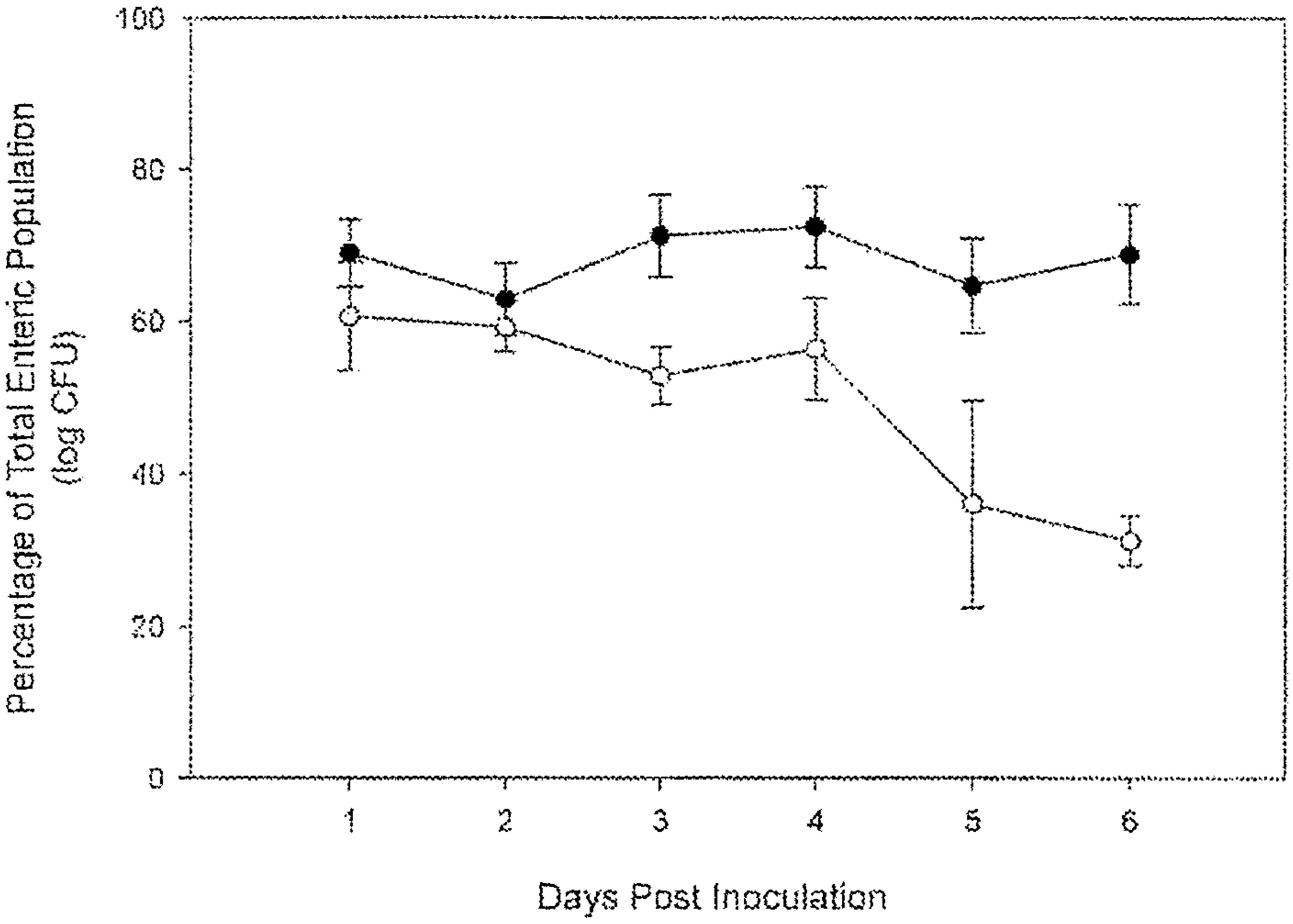

100
80
60
40
20
0
Percentage of Total Enteric Population
(log CFU)
1
2
3
4
5
6
Days Post Inoculation

Figure 6

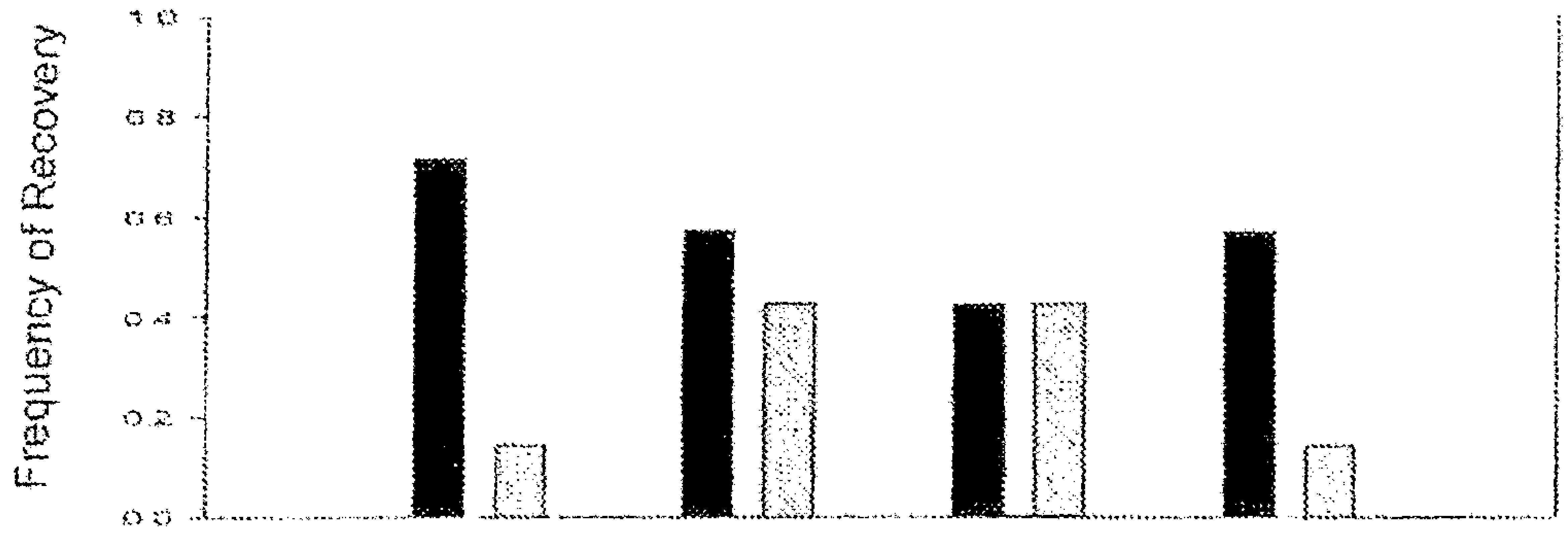


Figure 7A

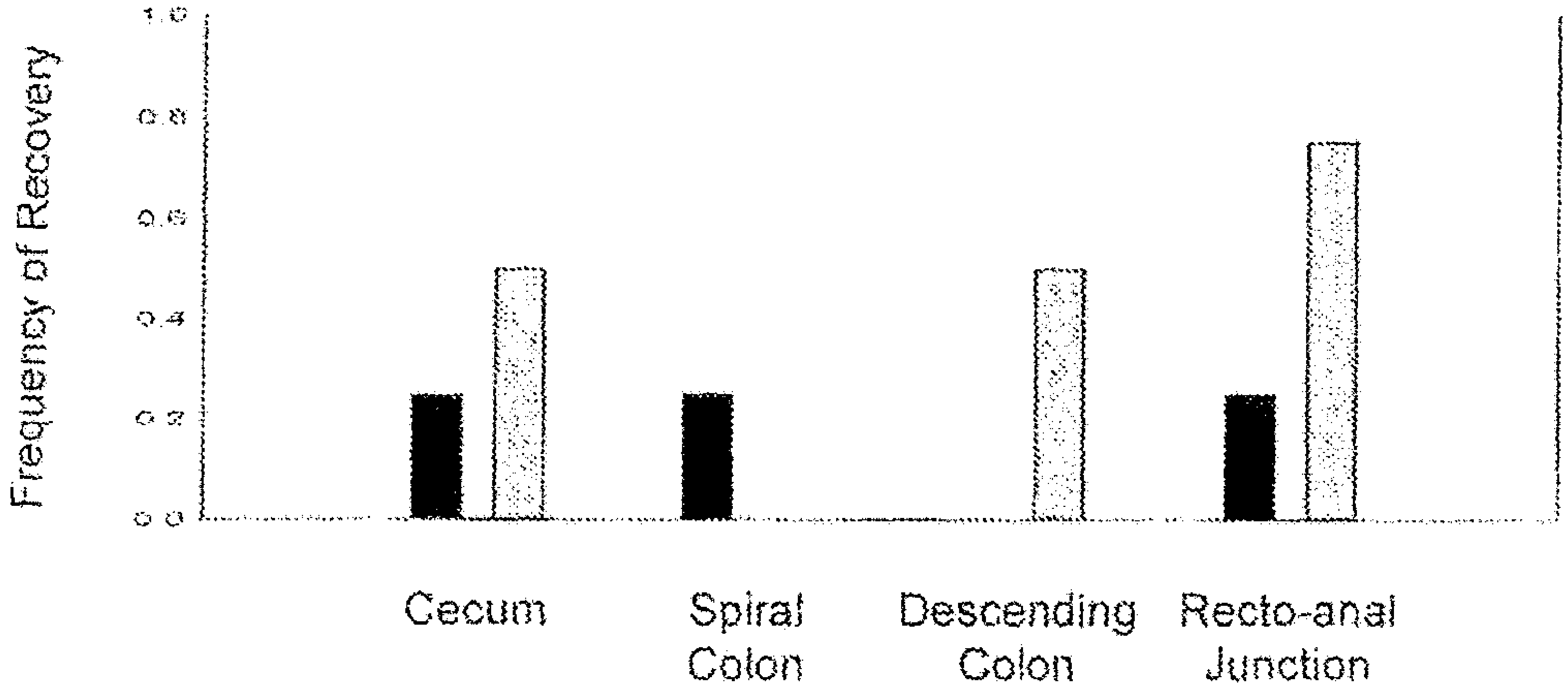
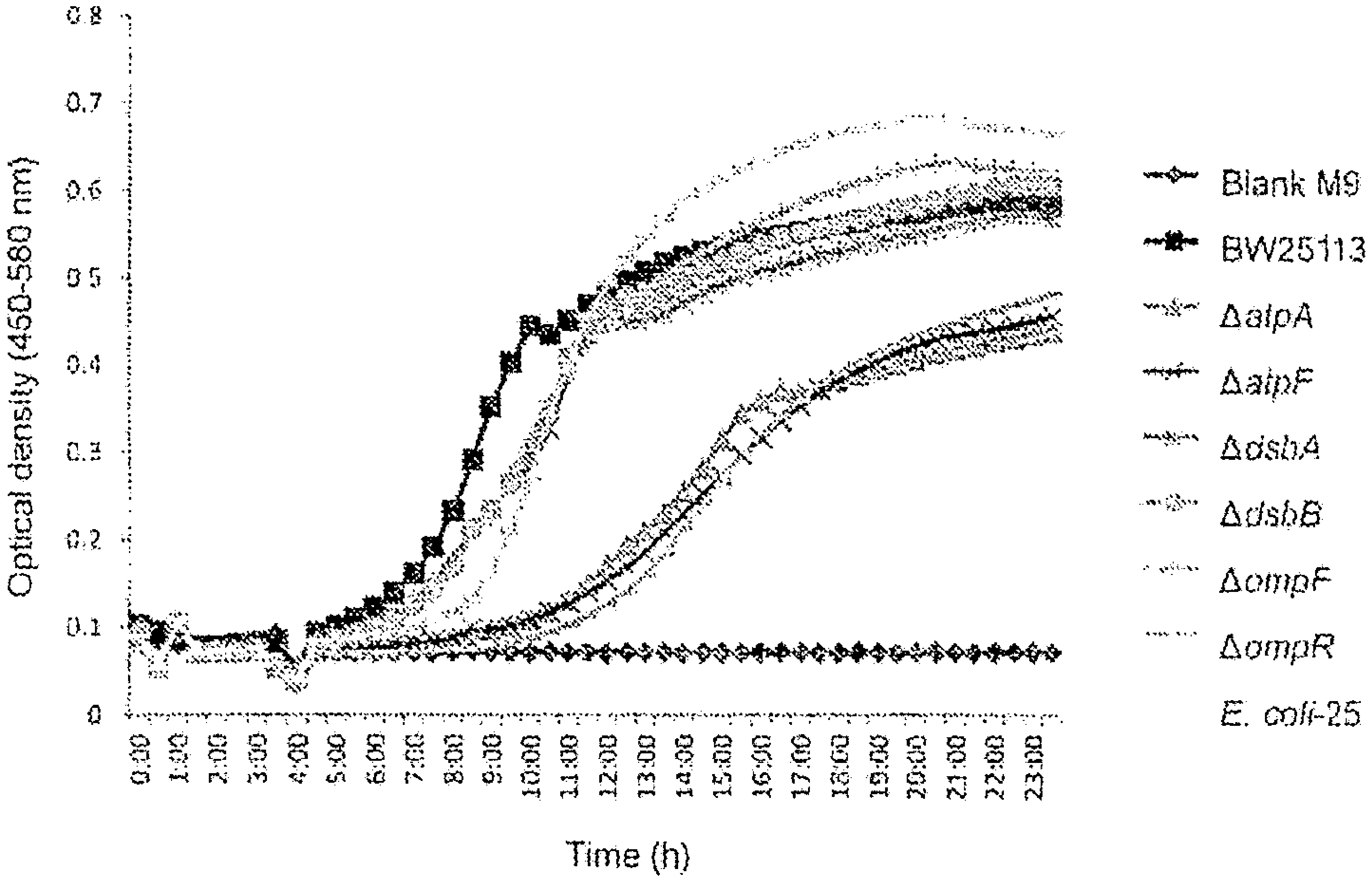
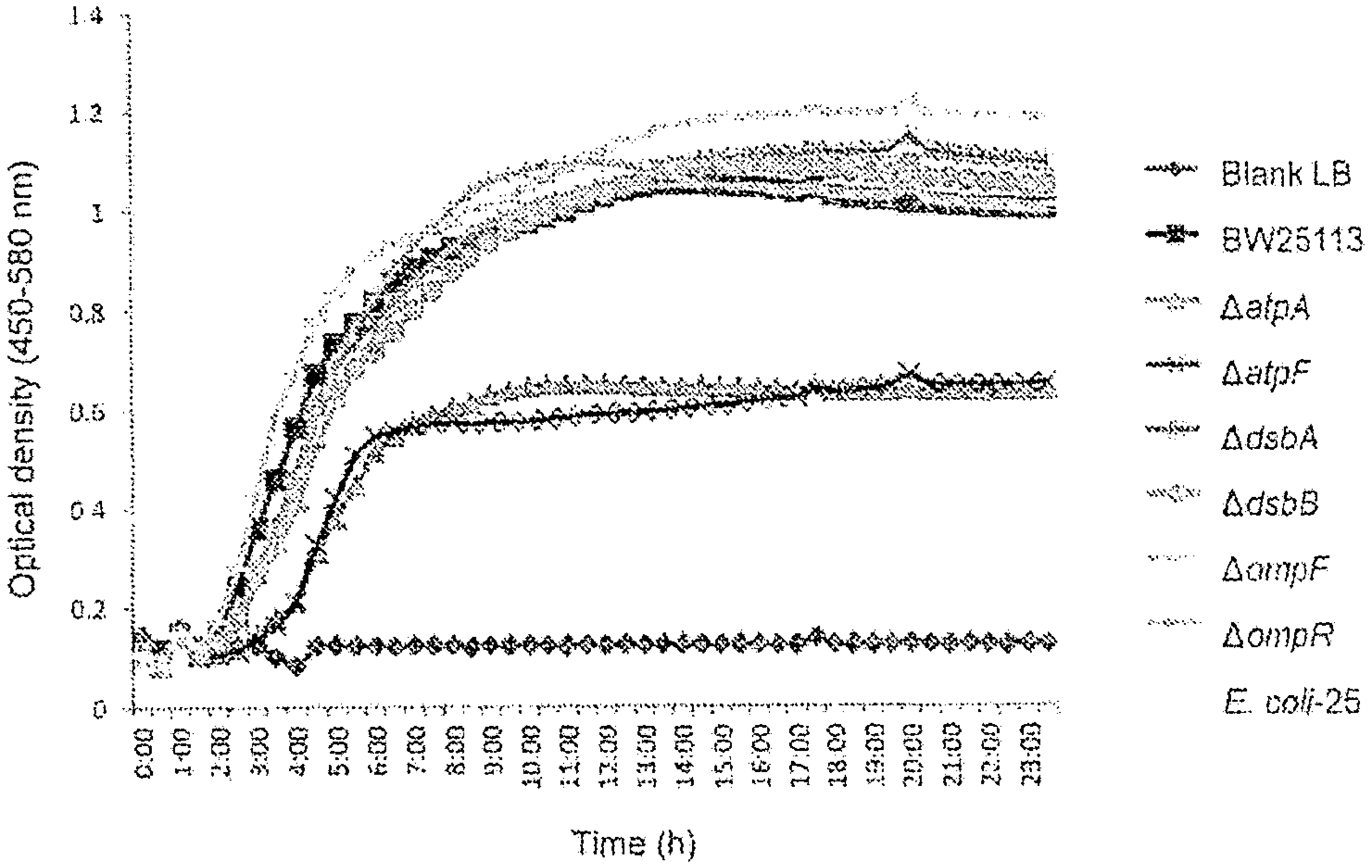


Figure 7B



Optical density (450-580 nm)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0:00
1:00
2:00
3:00
4:00
5:00
6:00
7:00
8:00
9:00
10:00
11:00
12:00
13:00
14:00
15:00
16:00
17:00
18:00
19:00
20:00
21:00
22:00
23:00
Time (h)
Blank M9
BW25113
ΔalpA
ΔalpF
ΔdsbA
ΔdsbB
ΔompF
ΔompR
E. coli-25

Figure 8A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1.2
1
0.8
0.6
0.4
0.2
0
Optical density (450-580 nm)
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5:00
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7:00
8:00
9:00
10:00
11:00
12:00
13:00
14:00
15:00
16:00
17:00
18:00
19:00
20:00
21:00
22:00
23:00
Time (h)
Blank LB
BW25113
ΔatpA
ΔatpF
ΔdsbA
ΔdsbB
ΔompF
ΔompR
E. coli-25

Figure 8B

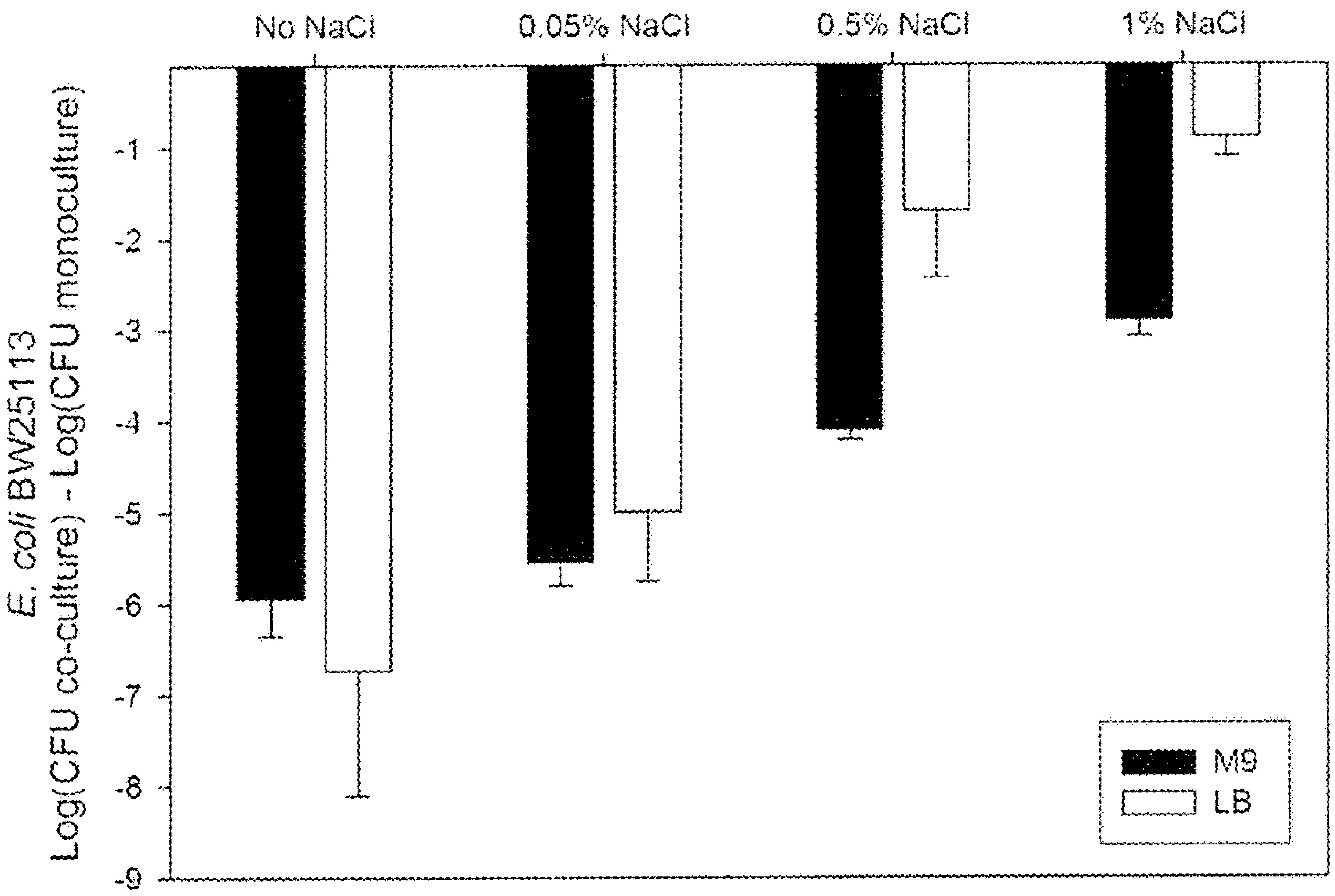


Figure 9A

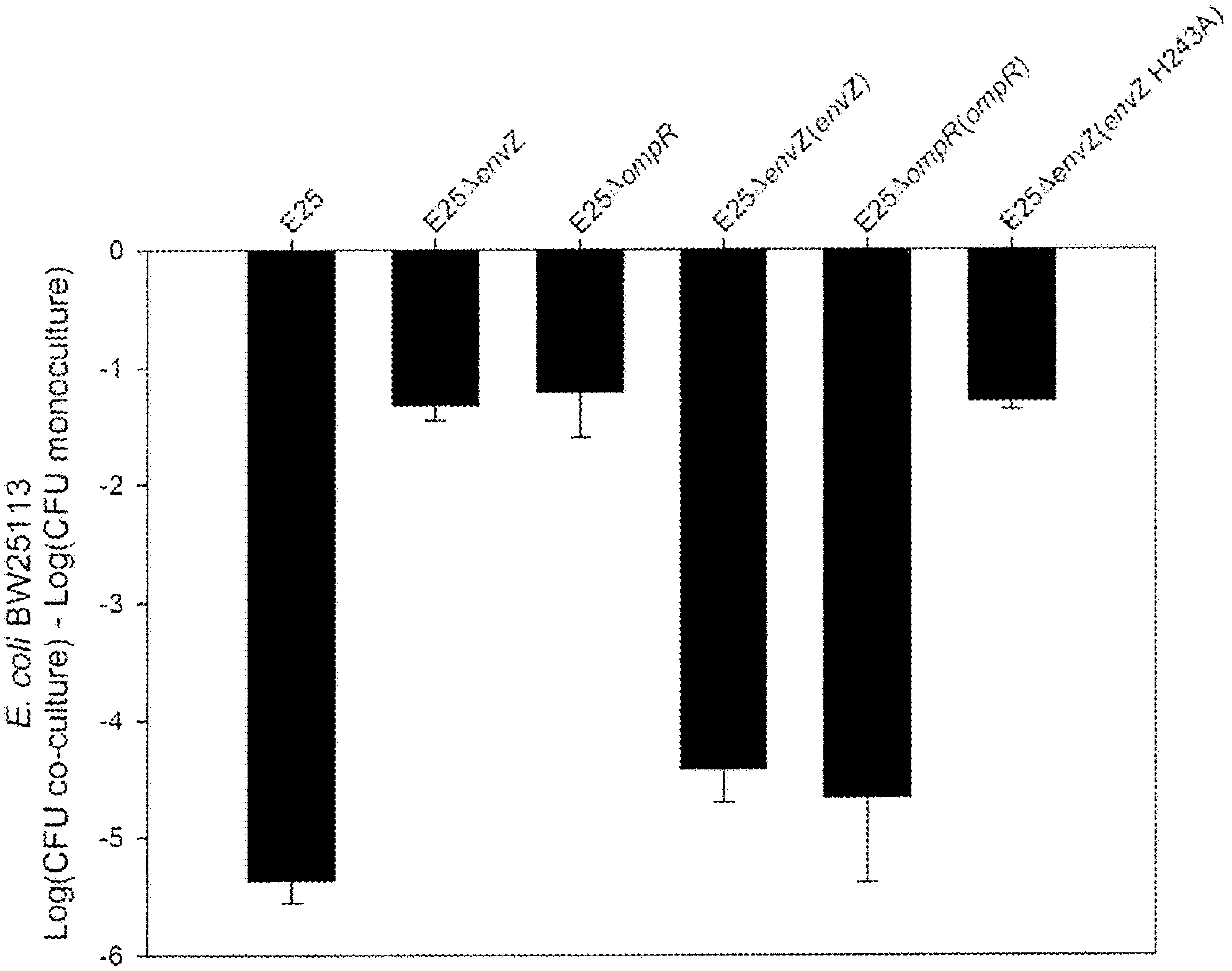


Figure 9B

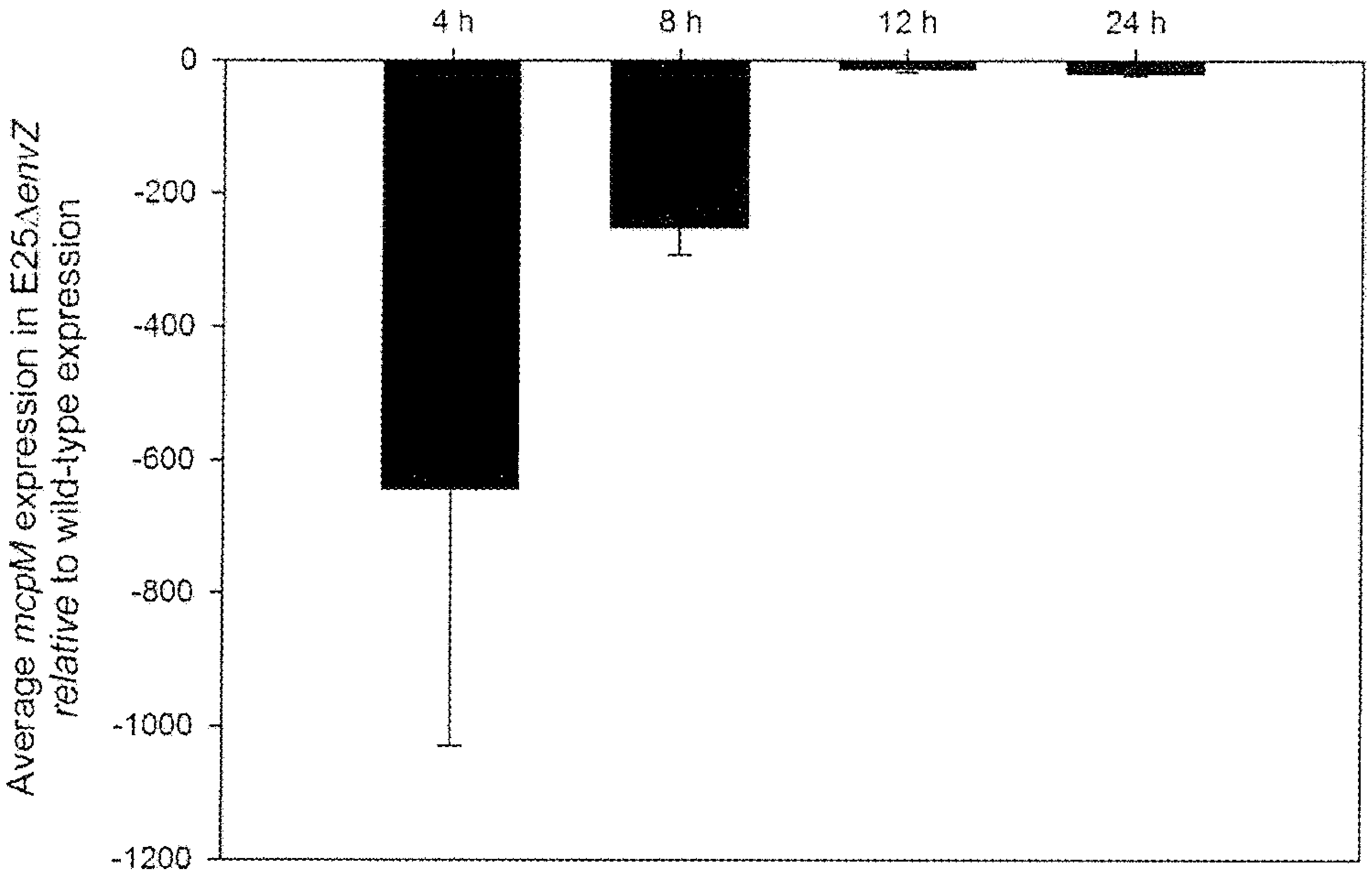


Figure 10A

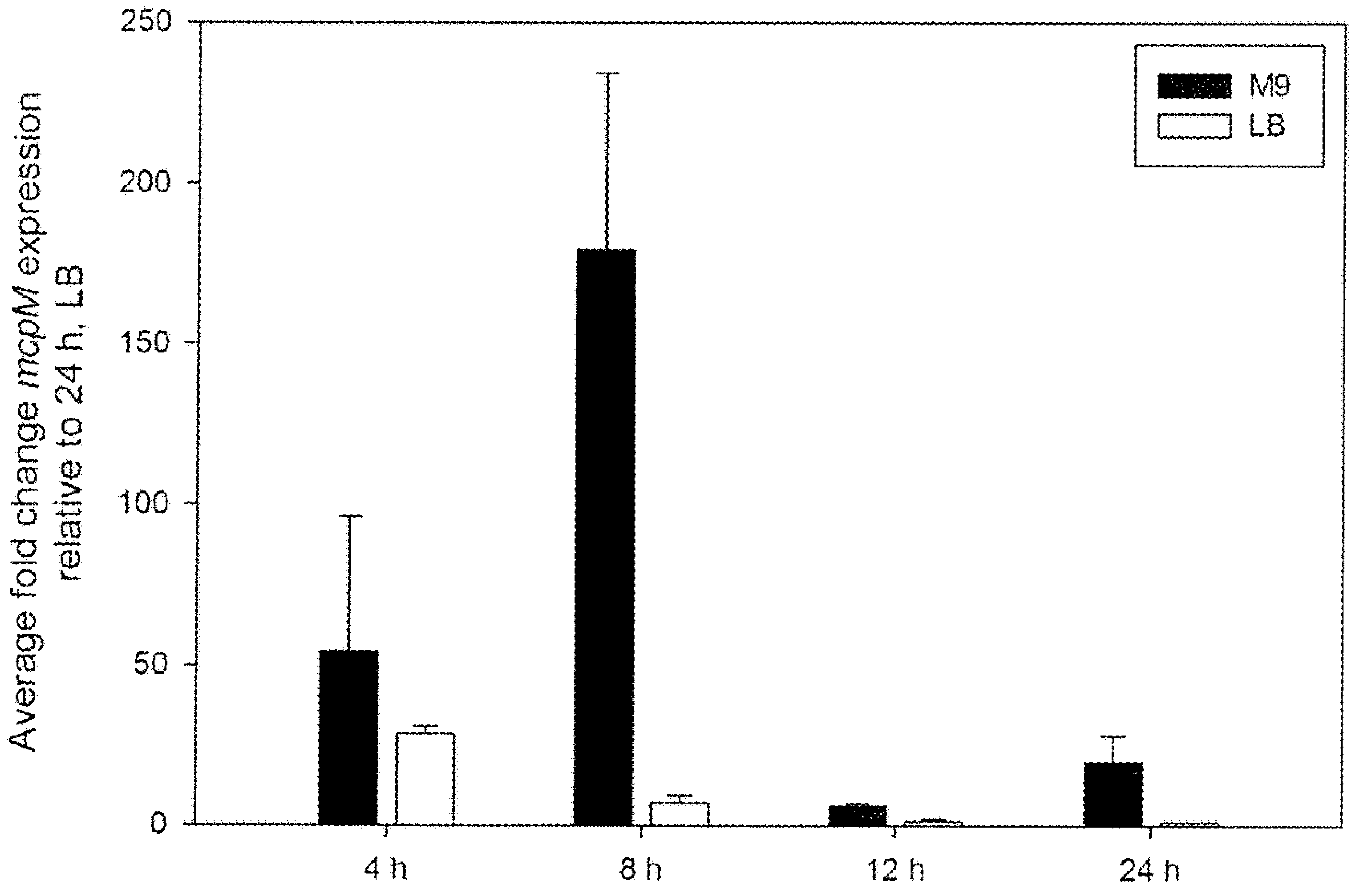


Figure 10B

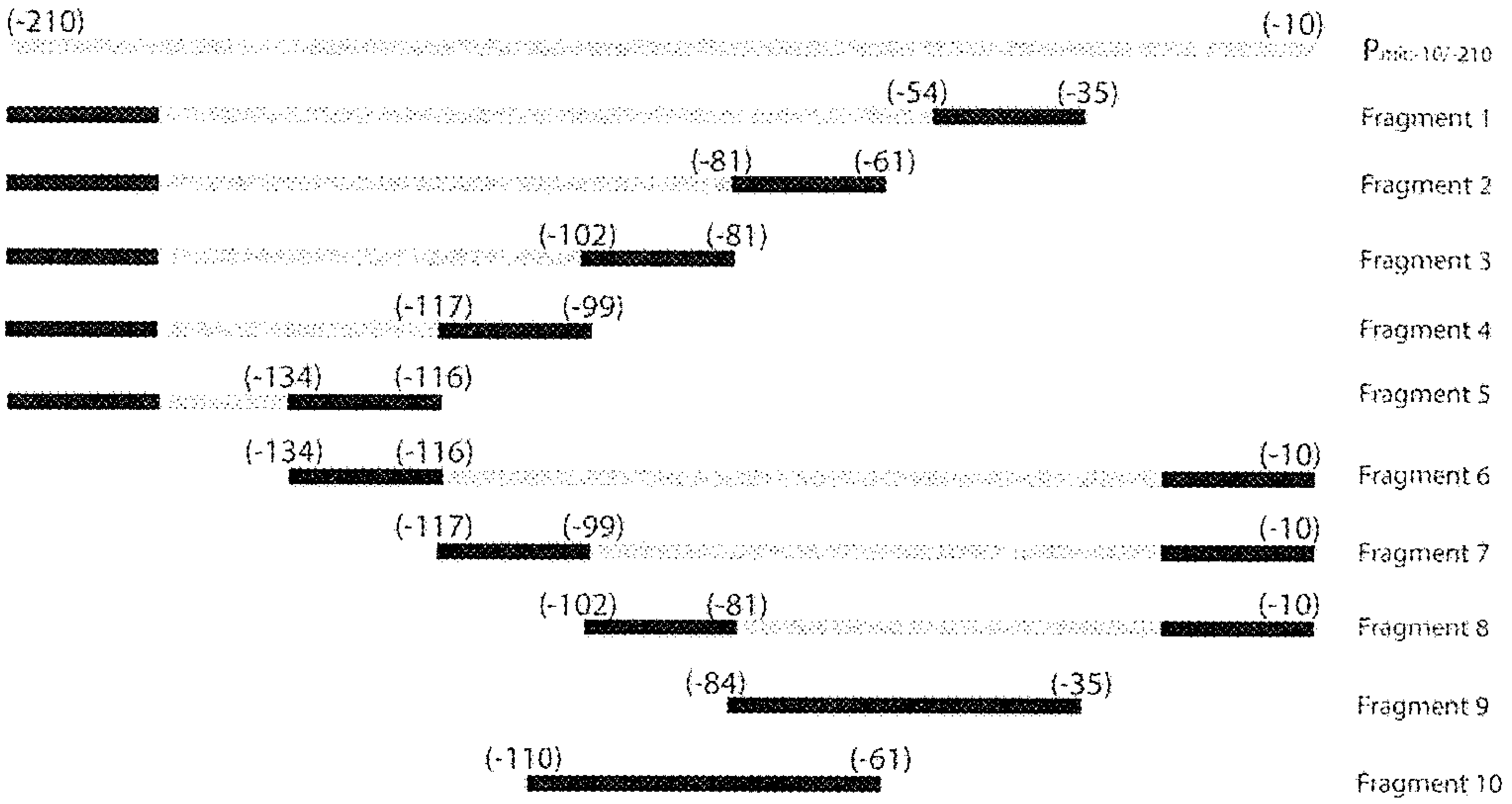
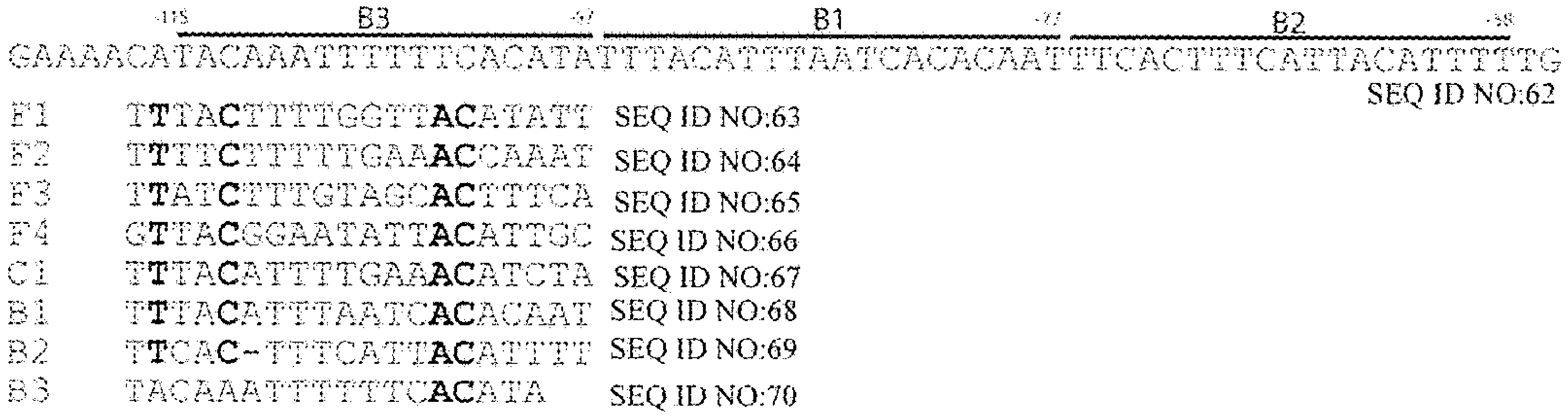


Figure 11A



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SEQ ID NO:62

F1	T**T**TA**C**TTTTGGTT**AC**ATATT	SEQ ID NO:63
F2	T**T**TT**C**TTTTTGAA**AC**CAAAT	SEQ ID NO:64
F3	T**T**AT**C**TTTGTAGC**AC**TTTCA	SEQ ID NO:65
F4	G**T**TA**C**GGAATATT**AC**ATTGC	SEQ ID NO:66
C1	T**T**TA**C**ATTTTGAA**AC**ATCTA	SEQ ID NO:67
B1	T**T**TA**C**ATTTAATC**AC**ACAAT	SEQ ID NO:68
B2	T**T**CA**C**-TTTCATT**AC**ATTTT	SEQ ID NO:69
B3	TACAAATTTTTTC**AC**ATA	SEQ ID NO:70

Figure 11B

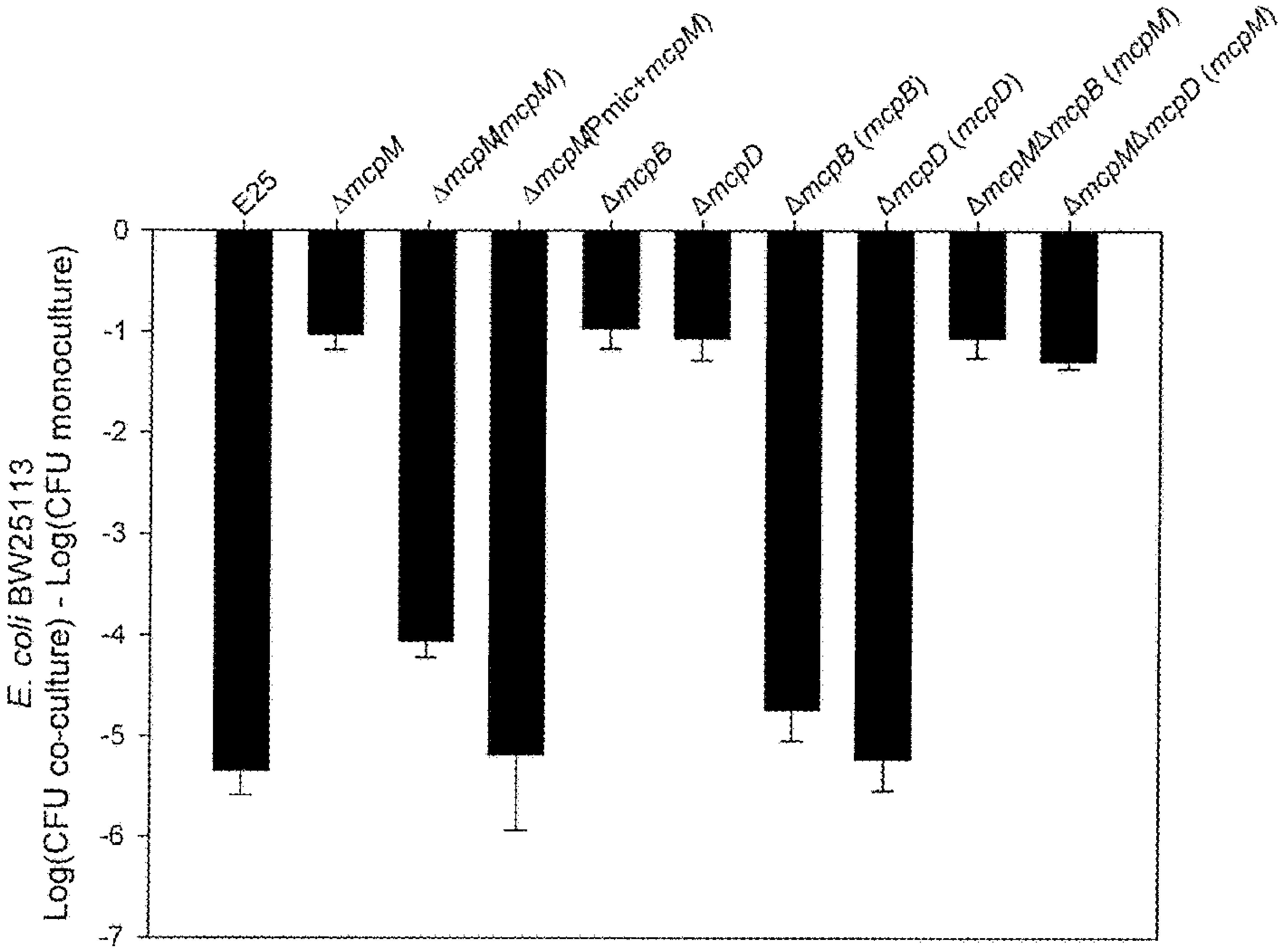


Figure 12

Figure 13

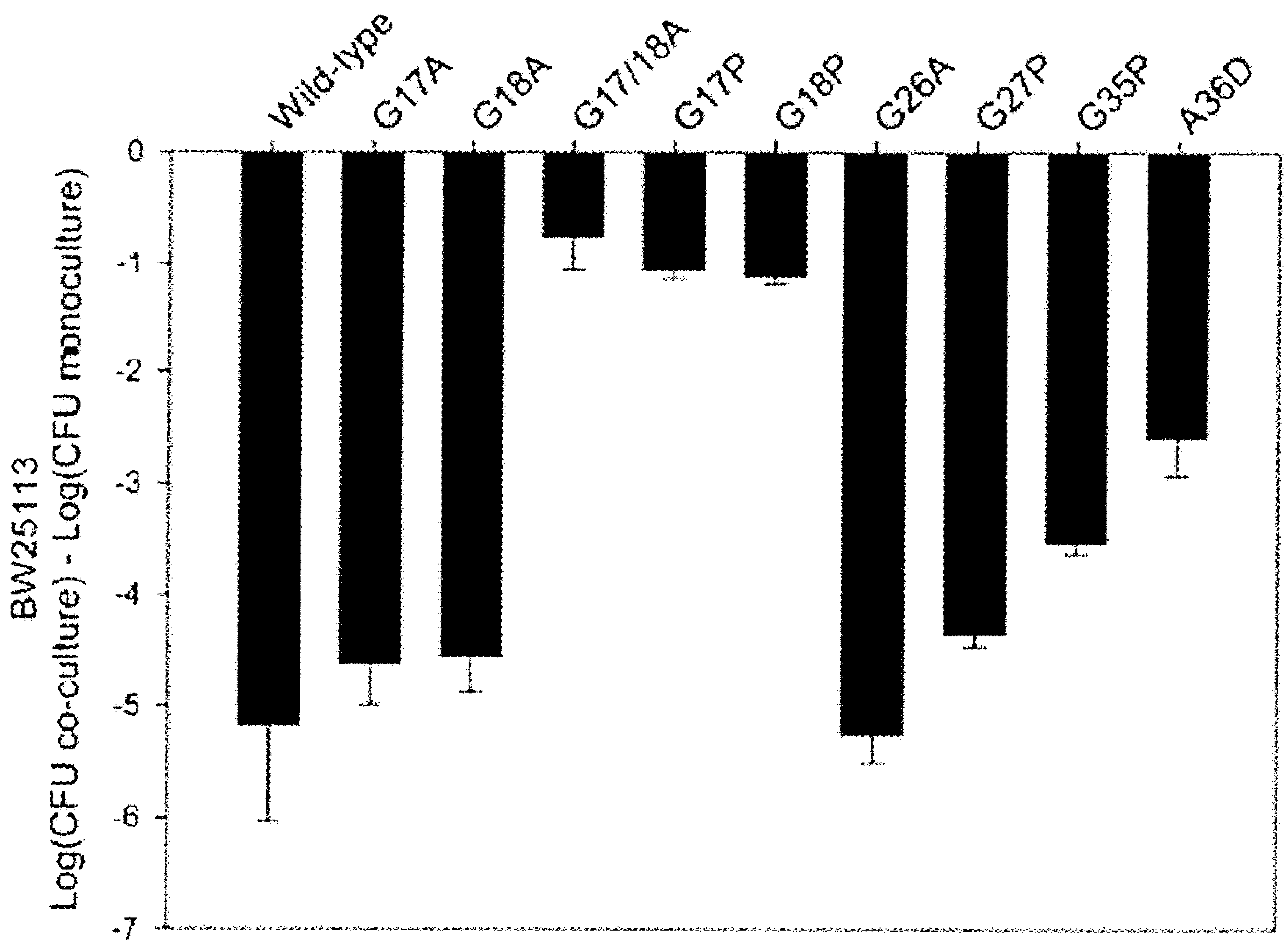


Figure 14

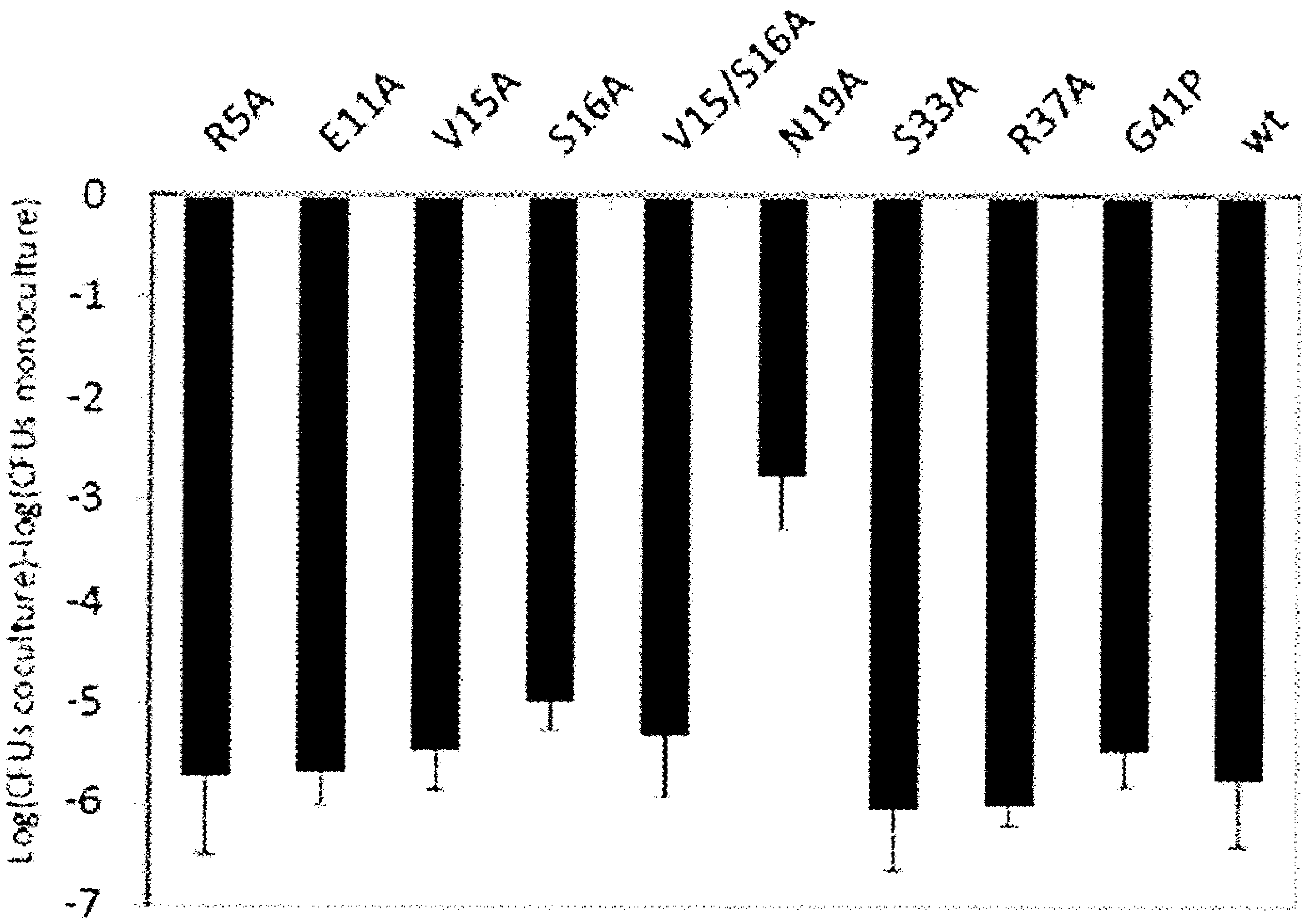


Figure 15

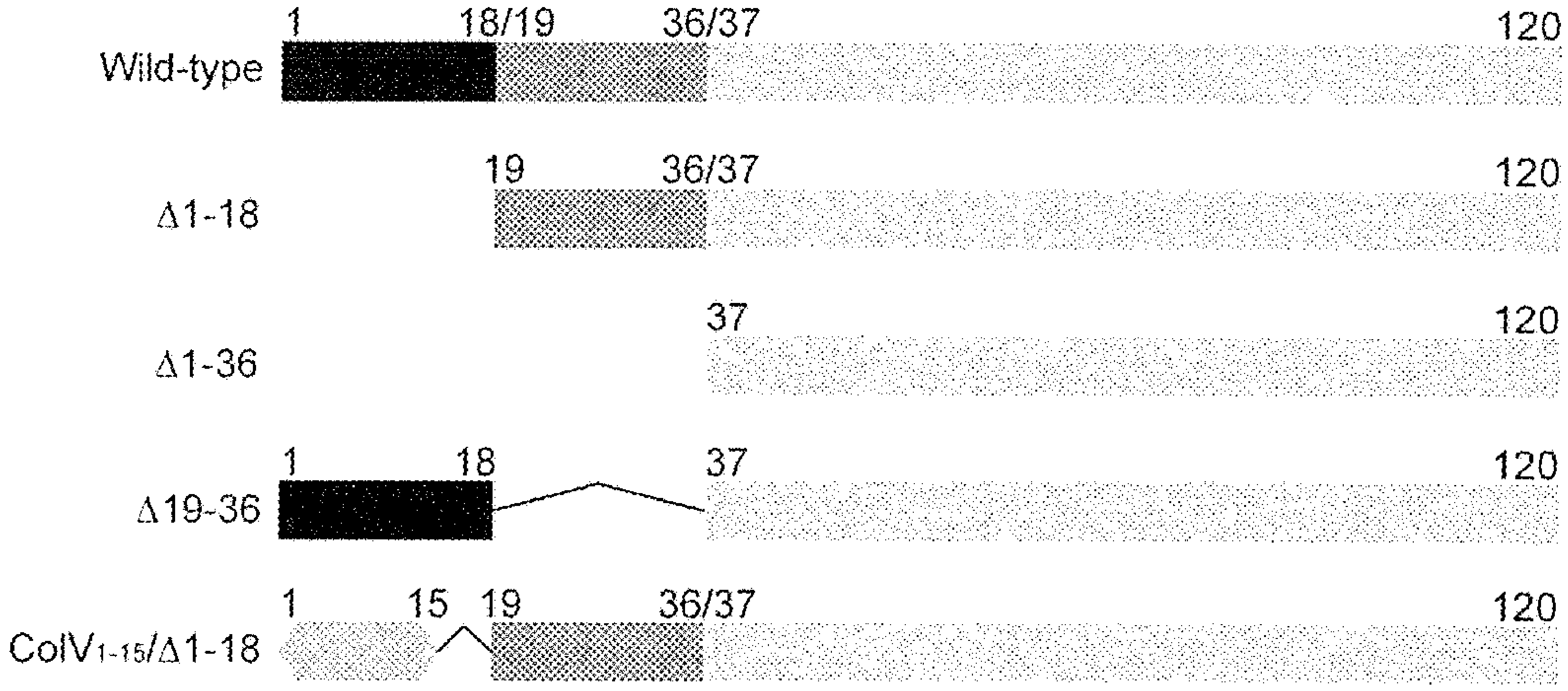


Figure 16A

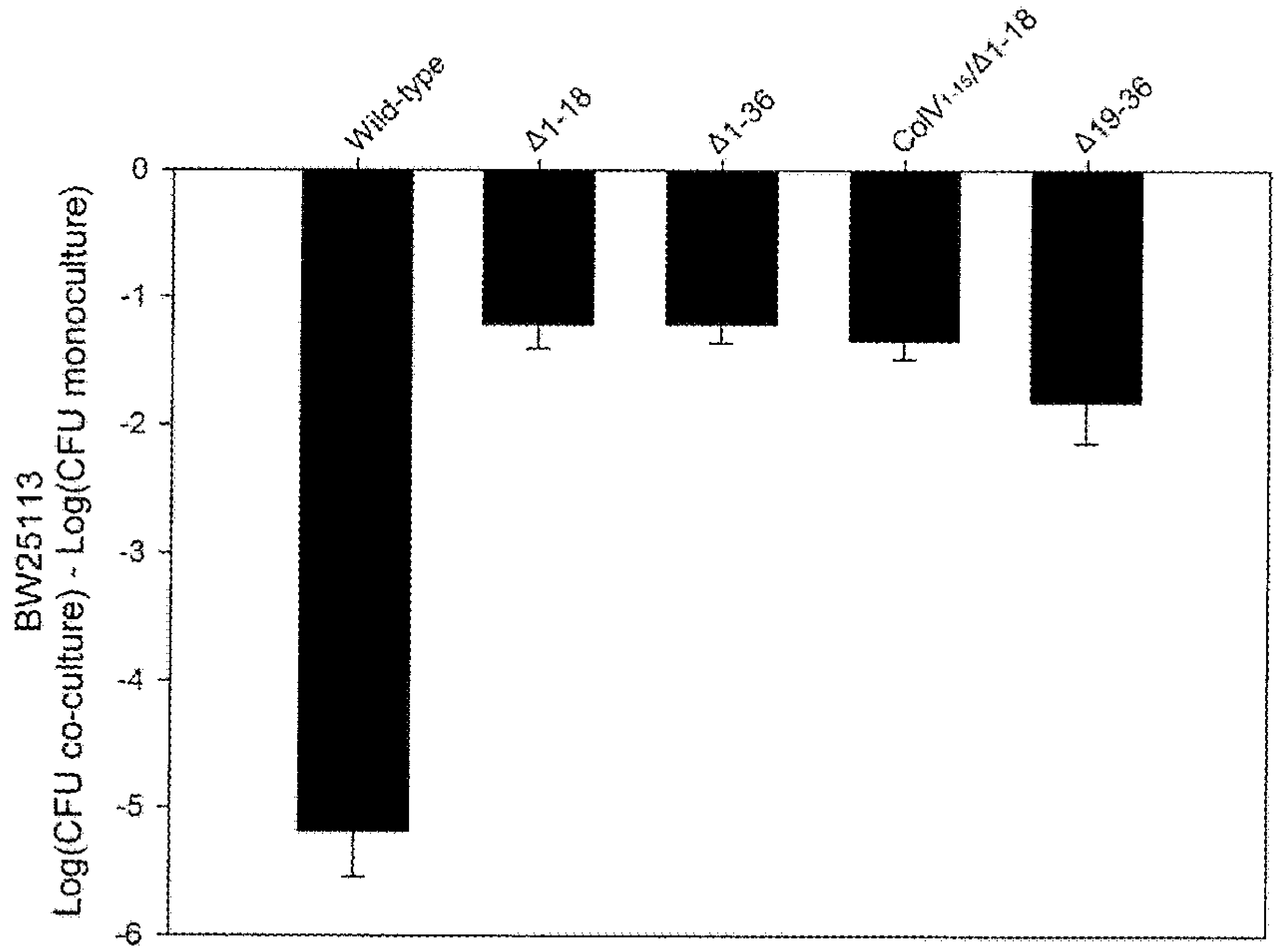


Figure 16B

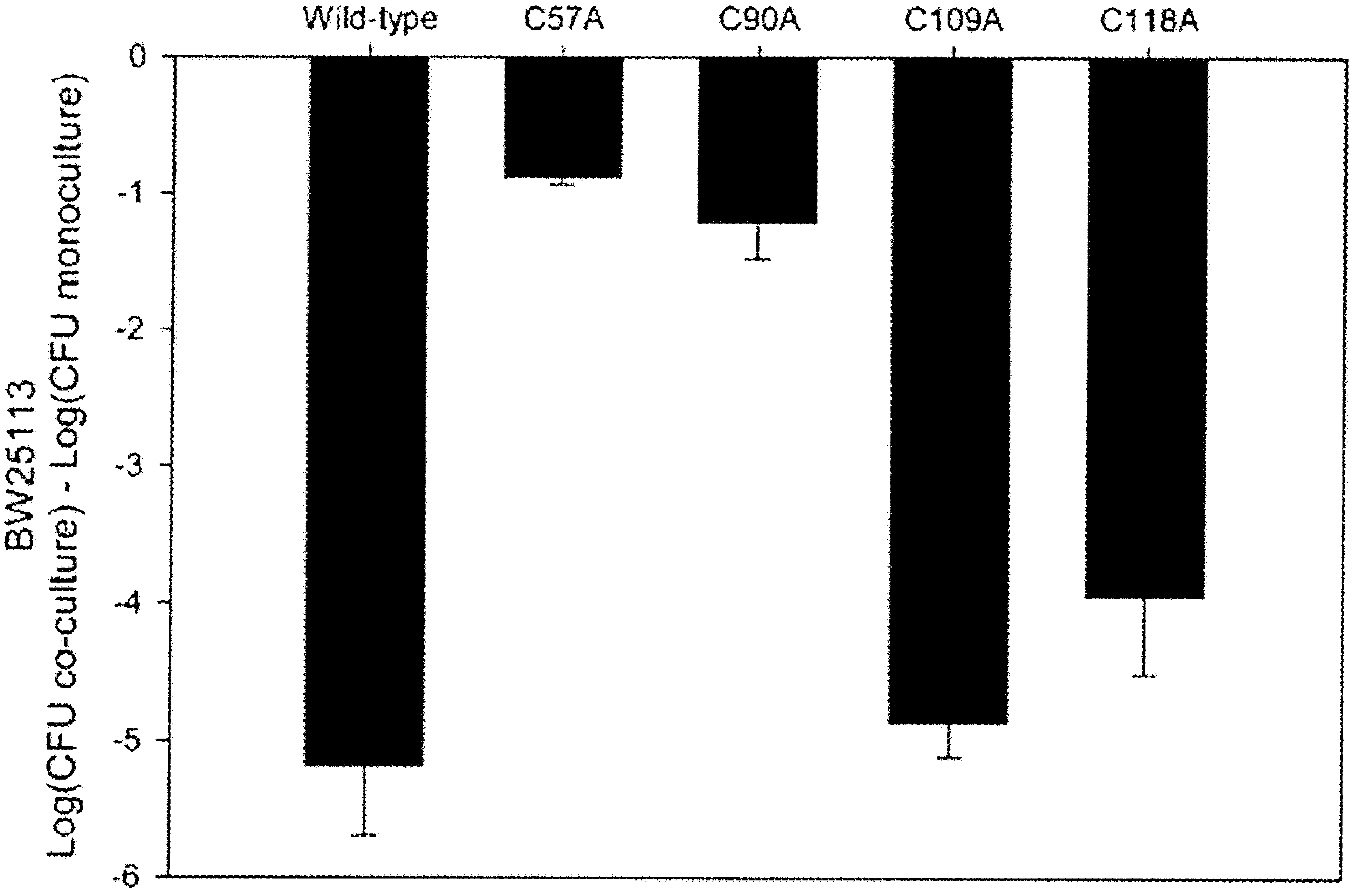


Figure 17

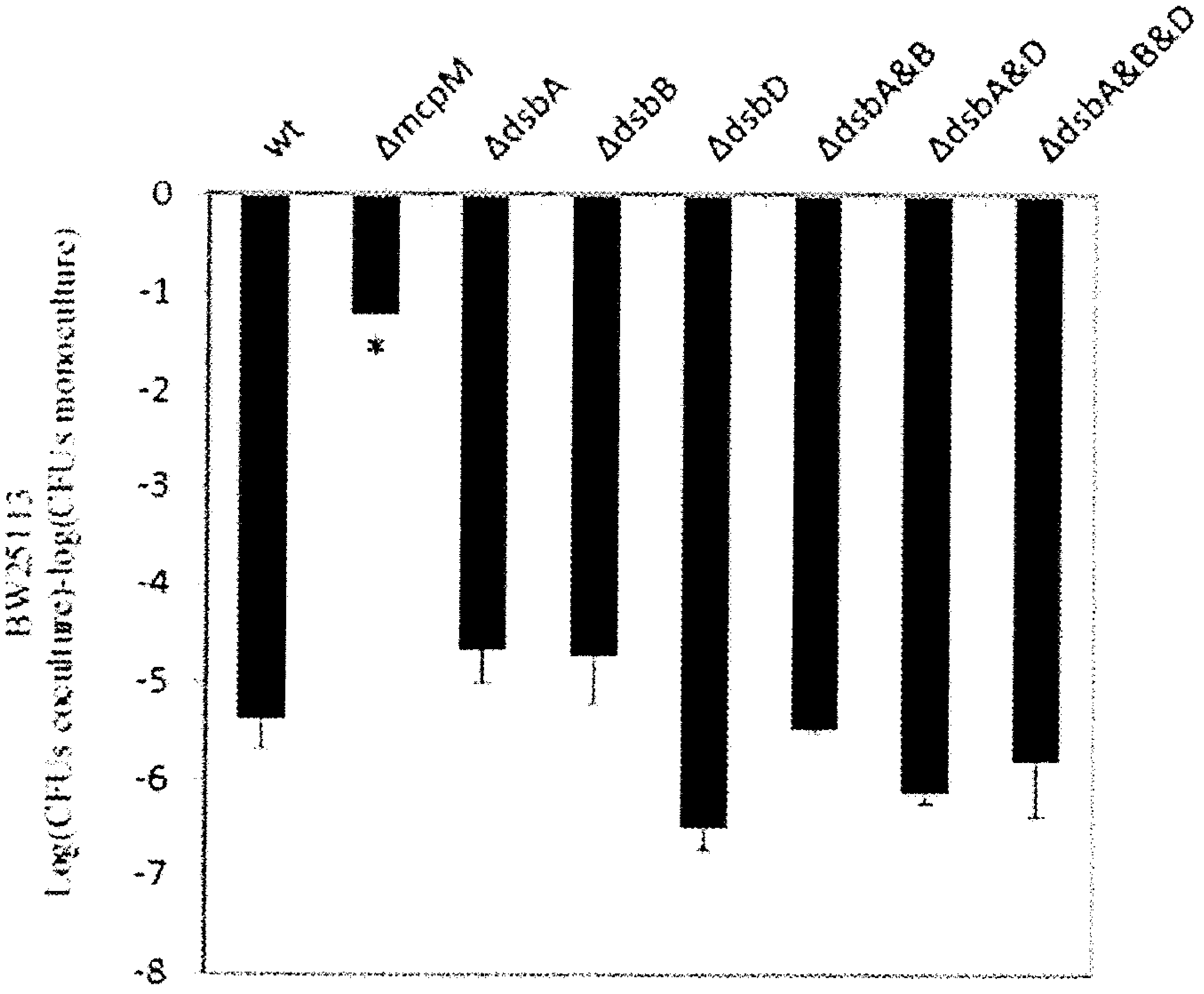


Figure 18

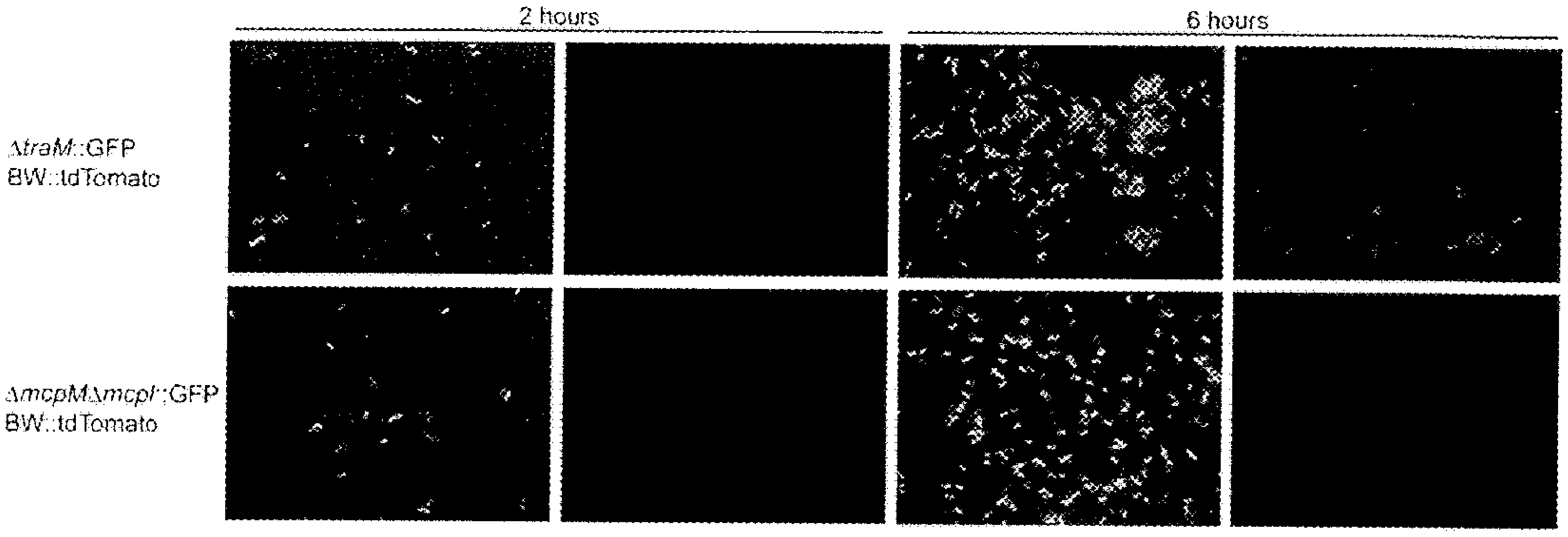
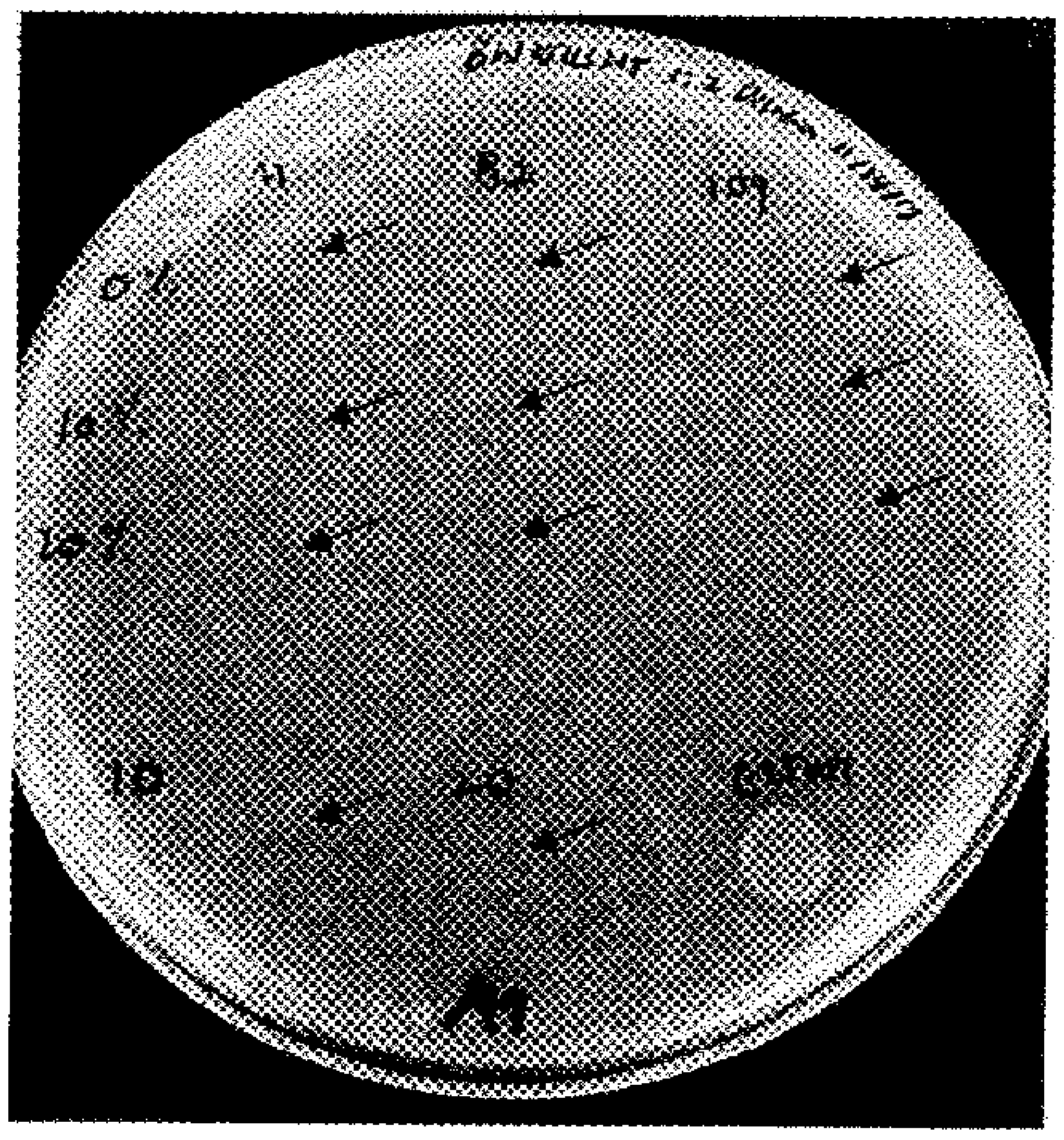


Figure 19



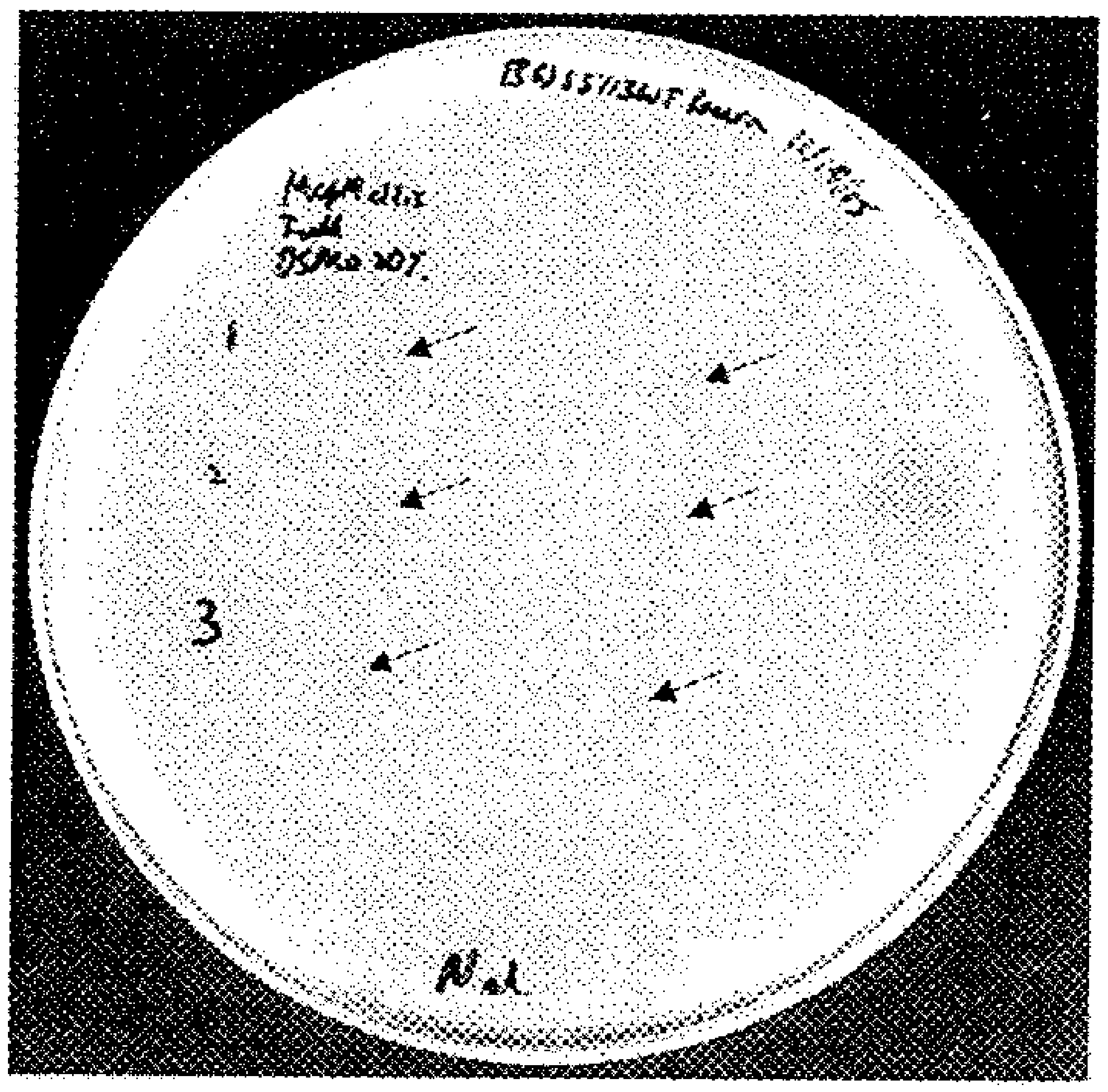


Figure 20A

Figure 20B

MICROCIN AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a divisional application of USSN 15/268,666 filed Sep. 19, 2016, now U.S. Pat. No. 10,022,423, which was a Continuation-in-Part of USSN 14/407,975, filed Dec. 15, 2014, now U.S. Pat. No. 9,492,500, which was a National Stage Application based on the International Application No. PCT/US2013/045937 filed Jun. 14, 2013 which claims priority to U.S. Provisional Application 61/660,616 filed Jun. 15, 2012 .

This invention was made with government support under Grant Number 2011-67005-30021 awarded by the United States Department of Agriculture through the National Institute of Food and Agricultures. The government has certain rights in this invention.

ACKNOWLEDGEMENT OF FEDERAL FUNDING

Particular aspects of the present invention were, at least in part, supported by Grant Number 2010-04487 from the United States Department of Agriculture (USDA-AFRI-NIFA) and Grant Number 10-086 from the National Pork Board. The United States government and National Pork Board therefore may have certain rights in the invention.

FIELD OF THE INVENTION

Aspects of the invention relate generally to bacteria, bacteriocins (e.g., colicins or microcins) and proximity-dependent inhibition (PDI), and in more particular embodiments to compositions and methods for controlling and/or killing pathogenic bacteria (e.g., enterohemorrhagic and/or enterotoxigenic strains of *E. coli*), comprising use of a novel microcin.

BACKGROUND

Escherichia coli are commonly found in the gut of both humans and animals. Most *E. coli* are considered symbiotic; however, pathogenic strains have been isolated that are associated with foodborne illness in people and animals e.g., pathogenic *E. coli* K88 and K99 affect swine and calves, respectively. Transmission of pathogenic *E. coli* occurs through fecal contamination of food or water, and is commonly associated with the consumption of under-cooked meat, raw milk, or contaminated vegetables.

Pathogenic *E. coli* includes the Shiga-toxin producing strains known as STEC. Shiga-toxin is named for its resemblance to the Shiga-toxin produced by *Shigella dysenteriae*. STEC infection can be asymptomatic, or include symptoms of fever, watery diarrhea, severe abdominal pain, hemolytic uremic syndrome (HUS) and even death, with more severe cases typically being reported in young children or the elderly. Enterohaemorrhagic *E. coli* (EHEC) are a subset of STEC, characterized by their ability to form attaching and effacing intestinal lesions. Cattle are the main reservoir for EHEC, the bacteria living asymptomatically in the cow intestine, although these bacteria have also been isolated from the intestinal tract of other domestic animals including sheep, pigs, goats, and dogs. These EHEC predominantly colonize the recto-anal junction of cattle, thereby increasing the risk of transmission to humans through fecal contamination. Numerous EHEC have been isolated including serotypes O111, O145, O103, O26, and O157. According to the Centers for Disease Control and Prevention, O157:H7 is the most common serotype that causing *E. coli*-linked food poisoning in the United States. The infectious dose is estimated to be as low as 10-100 bacteria. EHEC infections can be difficult to treat and some antibiotics actually worsen symptoms of an EHEC infection by inducing Shiga-toxin production and increasing the risk of HUS.

The introduction of antibiotics as therapeutics in the mid-1940s was an important advancement for medicine in terms of reducing human morbidity and mortality. The subsequent emergence of antibiotic resistant bacteria, however, indicates that bacteria adapt to antibiotic pressure. Resistance can be acquired and maintained within a population through horizontal transfer of resistant genes, and/or through selection for mutations that confer resistance. Unfortunately, the use of antibiotics is widespread and invariably selects for resistance as continual exposure to the drugs inhibit susceptible strains and allows resistant strains to emerge and dominate a population. Selection for resistance occurs for all bacteria exposed to antibiotics, not just the specific pathogens that are being targeted. For example, when enrofloxacin was used to treat *E. coli* infections in poultry, it simultaneously selected for resistance in *Campylobacter jejuni*, which is another important food-borne pathogen. The increasing prevalence of resistant bacterial pathogens threatens the effectiveness of currently available antibiotics and presents a difficult challenge in human and animal medicine. The development of novel strategies to control pathogenic bacteria is necessary to 1) combat infection by existing strains and 2) provide alternatives so that antibiotic use, and hence the emergence of resistant strains, can be decreased.

Some bacteria have developed the ability to inhibit other bacteria, and further characterization of how this occurs could be helpful in the design of new anti-bacterial strategies. For example, cell-cell inhibition mechanisms have been documented in the literature and range from contact-dependent inhibition (1, 20) to production of narrow-spectrum antimicrobial proteins called bacteriocins. Bacteriocins typically restrict the growth of closely related bacteria (reviewed in (28, 31)). *E. coli* produce numerous bacteriocins (31), classified as either colicins or microcins (2, 11). Colicins are high-molecular weight, whereas microcins are typically <10 kDa. Microcins can be either chromosomally or plasmid encoded, whereas colicins have only been found on plasmids (13, 29, 30). Colicin production is usually correlated with an SOS response to stress (22, 34) and release of the colicin typically occurs through cell lysis. Microcins are secreted from intact cells (8, 27). Bacteriocins have been identified that kill competitors through pore formation, nuclease activity, or by inhibiting protein synthesis (3, 23-25).

Sawant et al. recently described a novel bacterial inhibition phenotype whereby defined strains of *E. coli* from cattle are able to inhibit growth of other *E. coli* strains including several strains of enterohemorrhagic *E. coli* (EHEC) and enterotoxigenic *E. coli* (ETEC) (32). During in vitro competition assays, susceptible strains declined an average 4-6 log in population size relative to their expected population density when grown as monocultures. The inhibition phenotype was called "proximity-dependent inhibition" (PDI) because of the apparent need for inhibitor and susceptible strains to be located in close physical proximity for the phenotype to be observed. Two different *E. coli* strains were described as expressing this trait (PDI$^+$); multidrug resistant *E. coli*-25 and antibiotic susceptible *E. coli*-264. *E. coli*-25 and *E. coli*-264 do not affect the growth of each other, indicating that immunity is either conferred actively through the presence of an immunity mechanism, or passively through the absence of a receptor ligand found on susceptible cells.

Certain characteristics of the PDI phenotype resemble that of microcin production. For example, inhibition is effective against closely related species; PDI is not dependent on an SOS response; and production presumably does not kill the inhibitor strain (32). Nevertheless, microcins are soluble proteins and when Sawant et al. (32) employed a split-well experiment they demonstrated that close cell-cell proximity is required for the PDI phenotype to function. These findings suggest that the inhibition mechanism is not due to a soluble molecule unless the concentration is so low as to require close proximity to be effective (32).

The initial report of PDI provided a detailed description of the phenotype and a similar phenotype has been described between *Bibersteinia trehalosi* and *Mannheimia haemolytica* (4). Nevertheless, the exact mechanism of PDI and requisite genes for inhibition and immunity were not known at the time that the PDI was originally described. Progress in this field could aid the development of strategies to combat the emergence and spread of pathogenic bacteria, and to provide treatments for infection with pathogenic bacteria.

SUMMARY OF EXEMPLARY ASPECTS

Particular embodiments of the invention demonstrate, for the first time, that “proximity-dependent inhibition” (hereinafter “PDI”) results in death of the susceptible cells, and that PDI can be used for killing pathogenic *E. coli* in vitro on surfaces and materials of interest, and in vivo, and further the PDI can be used prophylactically and therapeutically.

Additional embodiments of the invention identify the PDI gene cluster, which resembles that of a class IIa microcin. The gene cluster includes ORFs putatively encoding proteins for microcin synthesis, immunity, and export. In addition, tolC is required for inhibition, thereby confirming that the microcin is secreted by a type I secretion system (T1SS).

According to further embodiments of the invention, the PDI phenotype is caused by a novel microcin, designated herein as MccPDI, and MccPDI is utilized in a number of different and beneficial applications. In some instances, the use of MccPDI and/or bacteria that produce MccPDI advantageously replaces the use of antibiotics.

Every strain from a genetically diverse panel of *E. coli* O157:H7 (n=25) and additional strains of *E. coli* serovar O26 were susceptible to the PDI phenotype. Live-dead staining was consistent with inhibition by killing of susceptible cells. Comparative genome analysis identified the genetic component of PDI, which is composed of a plasmid-borne (Incl1) operon encoding a putative microcin and associated genes for transport, immunity, and microcin activation. Transfer of the plasmid to a PDI$^-$ strain resulted in transfer of the phenotype and deletion of the genes within the operon resulted in loss of the inhibition phenotype. Deletion of chromosomally encoded tolC also resulted in loss of the inhibitory phenotype and this confirmed that the putative microcin is most likely secreted via a type I secretion pathway. Deletion of an unrelated plasmid gene had no effect on the PDI phenotype. Quantitative RT-PCR demonstrated that microcin expression is correlated with logarithmic-phase growth.

According to yet further embodiments of the invention, the ability to inhibit a diversity of *E. coli* strains indicates that this microcin has utility to influence gut community composition (Eberhart, L J, J N Ochoa, T E Besser, and D R Call. 2014. Microcin mccPDI reduces the prevalence of susceptible *Escherichia coli* in neonatal calves. Journal of Applied Microbiology doi: 10.1111/jam.12535), and substantial utility for control of important enteric pathogens.

In some aspects, the bacteria that are killed (lysed, inhibited, damaged, etc.) are any that have (carry, bear, include, contain, etc.) the OmpF protein in or as a component of their outer membrane. OmpF or “outer membrane protein F”, (or OmpF porin), is an integral membrane protein located in the outer membrane of *E. coli* bacteria. OmpF porin is found in a trimer formation and is a non-specific transport channel that allows passive diffusion of small, polar molecules (600-700 Da in size) through the cell’s outer membrane, e.g. water, ions, glucose, and other nutrients as well as waste products. Without being bound by theory, the microcin described herein appears to bind to OmpF when exerting its lethal effects.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1**. PDI is effective against a broad panel of O157:H7 and STEC O26 *E. coli* isolates. On average, susceptible populations were reduced greater than 5-logs following 24 h co-culture with *E. coli*-25. Clinical and bovine-biased O157 genotypes are indicated (33). *Klebsiella pneumonia* was not inhibited by PDI and is included as a negative control. Error bars correspond to the standard error of the mean based on duplicate experiments.

FIG. **2**. Schematic of the putative PDI sequence (~5 kb). Whole genome sequencing identified a large plasmid containing a unique region that is present in PDI$^+$ strains but not PDI$^-$ strains (GenBank accession JQ901381) (SEQ ID NO:). Bioinformatics identified five open reading frames putatively corresponding to genes for microcin synthesis (mcpM and mcpA), immunity (mcpI), and export (mcpD and mcpB).

FIGS. **3**A-B. Targeted gene deletion results in the loss of the PDI phenotype. A. CFUs of PDI$^-$ *E. coli*-186 following co-culture with wild-type *E. coli*-25 or *E. coli*-25 knockout mutants. Results are expressed as the difference in CFUs of the sensitive strain grown in co-culture and monoculture. B. Competitions with PDI$^+$*E. coli*-264 indicate which knockout mutants no longer exhibit immunity to PDI. Immunity to PDI is restored in the mcpI complemented clone. Results are expressed as the difference of log CFUs during co-culture and individual culture. Experiments were conducted in triplicate with error bars representing the standard error of the mean. *, statistically significant ANOVA (p-value <0.01 with Dunnett’s upper one-sided multiple-comparison test with control).

FIG. **4**. Expression of mcpM by *E. coli*-25 is correlated with growth phase. Expression of mcpM was measured during 24 hr culture in M9. Closed circles are the mean normalized mcpM expression±SEM (n=2 replicates). Open circles are the mean colony forming units (CFU)±SEM (n=2 replicates).

FIG. **5**. MccPDI-producing *E. coli*-25 inhibits the growth of susceptible *E. coli*-186 in neonatal calves. A competition index (CI) was calculated as $(X-Y)/(X+Y)$, where X is the CFU of *E. coli*-25ΔmcpMΔmcpI or *E. coli*-25ΔtraM colonies and Y is the CFU of *E. coli*-186 colonies. A CI approaching +1 indicates the *E. coli*-25 mutant is the dominant strain and a CI approaching −1 indicates *E. coli*-186 is dominant. The mean CI for the positive (black bars; *E. coli*-25ΔtraM and *E. coli*-186; n=7)) and negative control (white bars; *E. coli*-25ΔmcpMΔmcpI and *E. coli*-186; n=4) calves on day one and day six. Error bars correspond to the standard error of the mean and the asterisk shows significant difference between MccPDI-producing and non-producing groups (P=0.003).

FIG. **6**. The production of MccPDI contributes to the fitness of *E. coli*-25 within a calf. Each data point represents the percentage of the *E. coli*-25 mutant relative to the total lactose fermenting enteric CFU at the corresponding day post inoculation. Closed circles represent *E. coli*-25ΔtraM (n=7 calves) and open circles correspond to *E. coli*-25ΔmcpMΔmcpI (n=4 calves). Error bars represent the standard error of the mean.

FIGS. **7**A and B. MccPDI-producing *E. coli*-25 is recovered more frequently from GI tissues. Bars represent the frequency of recovery for competing strains in each trial at four segments of the GI tract: A. MccPDI-producing *E. coli*-25ΔtraM (black) and *E. coli*-186 (grey), B. MccPDI-knockout *E. coli*-25ΔmcpMΔmcpI (black) and *E. coli*-186 (grey).

FIGS. **8**A and B. Growth curves of *E. coli*-25, *E. coli* BW25113, and the MccPDI-resistant mutants ΔatpA, ΔatpF, ΔdsbA, ΔdsbB, ΔompF, and ΔompR when cultured in M9 minimal media (A) or LB media (B).

FIGS. **9**A and B. The EnvZ/OmpR two-component regulatory system controls the MccPDI phenotype. (A) Competition assays between an MccPDI-producing strain (*E. coli*-25) and a target strain (BW25113) in M9 and LB with different concentrations of NaCl for 12 h. Results are expressed as the difference of mean log CFU during co-culture and mono-culture (n=3 independent replicates; error bars=SEM). (B) ΔenvZ or ΔompR strains no longer exhibit the MccPDI phenotype. Results are shown for competition assays between different knockouts or their complemented strains and BW25113 or BW25113 (vector control). Results are expressed as the difference of BW25113 log CFUs during co-culture and mono-culture for 12 h (error bars=SEM; 3 independent replicates). For complementation experiments the competition assays were performed in M9 with 34 ug/ml chloramphenicol and 0.5 mM IPTG. *, significant ANOVA followed by a Dunnett's one-way multiple comparisons test versus control group (E25) (P<0.001).

FIGS. **10**A and B. Transcriptional analysis (qPCR) of mcpM for *E. coli*-25 and ΔenvZ strains in M9 media (error bars=SEM; **2** independent replicates). P<0.05 for all time points E25ΔenvZ versus WT at 8 h. GLM ANOVA followed by a Bonferroni multiple-comparison test. (B) Transcriptional analysis (qPCR) of nicpM for *E. coli*-25 cultured in LB or M9 media. Fold change is expressed relative to mcpM expression in LB at 24 h (error bars=SEM; 3 independent replicates).*P<0.01 based on ANOVA.

FIGS. **11**A and B. McpM promoter region has multiple binding sites for phosphorylated OmpR. (A) Diagram of the 10 DNA fragments that were selected from within the promoter region of mcpM. Fragments 1-8 were obtained by PCR amplification with bars indicating the primer position. Fragments 9-10 were obtained by annealing complementary oligonucleotides. (B) Three putative OmpR binding sites (B1, B2 and B3) are shown. F1, F2, F3 and F4 are the OmpR binding sites from the promoter region of *E. coli* ompF and C1 is the OmpR binding site identified with the promoter region of *E. coli* ompC.

FIG. **12**. CFU counts for *E. coli* BW25113 following competition with microcinproducing *E. coli*-25 and associated gene knockout and complemented strains. Results are expressed as the difference in CFUs of the sensitive strain grown in co-culture and monoculture (error bars=SEM; 3 independent experiments).

FIG. **13**. Amino acid sequence alignment of class II microcin precursors. MccPDEI (SEQ ID NO:24); McsS, microcin S (YP_006954535; SEQ ID NO:71); ColV, colicin V (CAA40746; SEQ ID NO:72); MccL microcin L (AAP03989; SEQ ID NO:73); Mcc24, microcin 24 (AAA88772; SEQ ID NO:74); MccE492, microcin E492 (AAD04332; SEQ ID NO:75); MccH47, MchB protein (CAB54534; SEQ ID NO:76). The arrows indicate the cleavage sites corresponding to McpM that we identified in this study. The inverted triangles indicate the position of the four cysteines of McpM. Sequence alignments were generated using ClustalX 1.83. Parenthetical numbers on the right indicate the amino acid position relative to the N terminus of each sequence.

FIG. **14**. Identification of McpM cleavage sites. Competition assays between the different site-specific mutants and BW25113 (vector ctrl). Results are expressed as the mean difference in CFU of the sensitive strain grown in co-culture and monoculture (error bars=SEM; 3 independent replicates). *, statistically significant ANOVA (P<0.01 with Dunnett's upper one-sided multiple-comparison test with control).

FIG. **15**. Competition assays between the different residue-specific mutants and BW25113 (vector ctrl). Competition was performed in M9 medium with chloramphenicol (34 ug/ml) and 0.5 mM IPTG for 12 h. Results are expressed as the difference in CFUs of the sensitive strain grown in co-culture and monoculture.

FIGS. **16**A and B. Loss of leader sequences blocks function of McpM. (A) Schematic diagram of different deleted constructs where residue numbering corresponds to amino acids in the full length McpM (wt). ColV1-15 is the signal peptide sequence from colicin V. (B) Competition assays between the different deleted mutants and BW25113 (vector control). Results are expressed as the mean difference in CFU of the sensitive strain grown in co-culture and monoculture (error bars=SEM; 3 independent replicates).

FIG. **17**. Cysteine residues 57 and 90 are required for McpM function. Competition assays between the 4 cysteine-residue mutants and BW25113 (vector control). Results are expressed as the difference in CFU of the sensitive strain grown in co-culture and monoculture (bars=SEM; 3 independent replicates). *, statistically significant ANOVA (P<0.01 with Dunnett's upper one-sided multiple-comparison test with control).

FIG. **18**. Disulfide bond formation system in *E. coli* is not involved into the mccPDI activity. CFUs of *E. coli* BW25113 following competition with wild-type E25 and different knockouts strains in the E25 background (single and double). Results are expressed as the difference in CFUs of the sensitive strain grown in co-culture and monoculture. Experiments were done in duplicate with error bars representing the standard error of the mean (SEM). *, statistically significant ANOVA (P<0.01 with Dunnett's upper one-sided multiple-comparison test with control).

FIG. **19**. MccPDI induces membrane permeability in susceptible cells. DAPI staining of the fluorescent-labeled strains in mono-culture and co-culture for 2 h and 6 h. The strains were derived from E25 (E25ΔtraM and E25ΔmcpMΔmcp I), and the MccPDI target strain BW25113. The DAPI staining was consistent with increased membrane permeability given exposure to MccPDI.

FIGS. **20**A and B. Recombinant McpM inhibits growth of susceptible *E. coli* independent of a McpM-producing *E. coli* strain. Plates containing (A) M9 agar media and (B) LB agar media are shown.

DETAILED DESCRIPTION

The genetic components that are responsible for the PDI phenotype observed from *E. coli*-25 and *E. coli*-264 are, for the first time, identified herein. This 4.8-kb cassette is present on pPDI (see Example 10 below) and is comprised of the genes mcpM, mcpI, mcpA, mcpD, and mcpB (see Example 9 below).

According to particular aspects, inhibition is mediated by the microcin encoded by mcpM, whose mature gene product is designated herein as MccPDI. The inventors demonstrate that a non-mccPDI strain called *E. coli*-82 is genetically very similar to *E. coli*-25 based on a technique called XbaI macrorestriction digest and pulsed-field gel electrophoresis. These two strains were sequenced using a 454 sequencing platform. The resulting sequences showed that the primary difference between the two strains was the presence of a large Incl1 plasmid. From this sequence, the inventors identified the mccPDI gene cassette. pPDI was subsequently marked with a kanamycin resistant cassette while simultaneously knocking out an unrelated genes, traM (ΔtraM). This plasmid was subsequently transferred to the PDI$^-$ strain, *E. coli*-4. Wild-type *E. coli*-4 does not exhibit the inhibitory phenotype, as indicated by competition assays with the susceptible *E. coli*-186 (Table 2; and see Example 6 herein below). *E. coli*-4 also exhibits susceptibility to PDI, based on CFU counts following co-culture with PDI$^+$*E. coli*-264. Following transformation with pPDIΔtraM, *E. coli*-4 acquired the inhibitory phenotype and immunity (Table 2; and see Example 6). Together these results indicate PDI and self-immunity are encoded by the 98.8 kb plasmid (see Example 10 below).

To demonstrate the necessity for each gene in the operon for PDI function, genetic knockouts were constructed and used herein to show that disrupting any gene within the PDI operon blocked the inhibitory phenotype and, additionally, immunity to PDI was lost in the mcpM and mcpI mutants (FIG. **3**). According to particular aspects, this indicated that all the genes were important for PDI, but immunity was dependent on only one or two genes. Due to the direct downstream location of mcpI, loss of immunity in the mcpM mutant was likely caused by a polar effect from ΔmcpM. This is consistent with the ability shown herein to complement immunity by the expression of mcpI alone. Although mcpI likely does not play a direct role in killing, it is necessary for self-immunity and for this reason is required for PDI. Deleting traM, a gene located ~20 Kb upstream of the PDI operon, did not affect either inhibition or immunity indicating that the methods used herein did not interfere with PDI. Furthermore, the tolC, mcpB, and mcpD mutants lost the ability to inhibit but retained immunity, consistent with a role in toxin transport.

According to particular aspects, and based on gene cluster and sequence analysis, MccPDI is best characterized as a Gram-negative class IIa microcin. The PDI gene cluster is relatively simple, consisting of two genes for export, one for immunity, one presumptively for microcin activation, and the microcin gene itself. Unlike class I and IIb microcins, which have several genes for post-translational modification, MccPDI only has one recognizable gene that is putatively required for microcin activation. The dedicated transport system involves the products of two plasmid-encoded genes, mcpB and mcpD. These two PDI genes have homology with hlyB and hlyD of the *E. coli* α-hemolysin T1SS (9). This multicomponent export system has similar organization to transport systems for other class II microcins, including MccE492, MccL, and MccV (10, 18, 26). McpB contains the transmembrane domains and nucleotide-binding domains, including the highly conserved Walker A and B motifs and ABC signature, characteristic of the ABC-transporter superfamily of proteins (21). McpD is thought to act as a membrane fusion protein, forming a channel through the periplasm and connecting to the outer membrane protein TolC, the third component of class II microcin export machinery (7, 10, 18, 26). In total, these proteins form the export system allowing secretion of protein from the cytoplasm across the periplasmic space and into the extracellular medium. McpM has homology to other microcin precursors within the N-terminal sequence, which encodes a putative signal peptide (6) that is consistent with T1SS transport. The presence of a conserved double glycine suggests the McpM precursor contains an 18-residue signal peptide that is cleaved to produce a mature MccPDI. There is no apparent sequence identity with other microcins in the activity region (C-terminal sequence) (6).

This indicates that a unique receptor is probably involved with the uptake of MccPDI and that the mechanism of killing is different from other microcins. The fact that only *E. coli* and *Shigella* (data not shown) are currently known to be susceptible to this PDI (MccPDI) suggests target cell recognition occurs through a specific receptor, possibly only expressed in these species.

Class IIa microcin gene clusters are typically composed of only four genes: two necessary for microcin export, one for immunity, and one encoding the microcin. The PDI operon is unique because it also includes a gene presumably involved with microcin processing or export. Deleting mcpA in *E. coli*-25 interrupts the inhibitory phenotype but does not affect immunity. It is possible this mutant has downstream effects on the microcin transport system (i.e. a polar effect); however, not to be bound by theory, bioinformatic analysis suggests the protein product is likely to be involved with post-translational modification of McpM. McpA has similarity to McmM and MceF (both 29% identity) of the MccM and MccE492 gene clusters, respectively. Wilkens et al. (36) showed an mceF mutant had a non-inhibitory phenotype and the microcin product collected from cell lysate was found at a higher molecular weight than mature MccE492, suggesting the presence of an inactive microcin precursor. Consistent with a role in protein modification, McpA contains a CaaX amino terminal protease domain (PF02517) that functions in post-translation modification of proteins with the CaaX sequence motif (35). Furthermore, the McpM C-terminus putatively contains a modified terminal CaaX sequence, suggesting McpM may be processed either before or during transport, resulting in the fully mature MccPDI.

Although microcins are released extracellularly to inhibit competing bacteria, no antimicrobial compounds were detected through membrane-divided competitions or spent media assays in previous work (32). It is possible the PDI microcin requires contact between competing cells or some other signal to become active in the media. Alternatively, the methods used in the Examples section herein may interfere with the detection of a soluble microcin in these assays, or the concentration of secreted microcin is too low to have a biological effect except when inhibitor cells express the microcin in close proximity to susceptible strains. Without being bound by theory, it is likely that MccPDI interacts with an outer membrane protein OmpF, which is highly conserved in all sequenced *E. coli* and *Shigella*. Data presented in Example 12 below shows that six genes (atpA, atpF, dsbA, dsbB, ompF, and ompR) were required for susceptibility to PDI, suggesting that OmpF acts as the receptor for MccPDI and the other genes required for sensitivity are necessary for expression and folding of OmpF, and/or they are required to translocate MccPDI across the cellular membrane.

Like other microcins, MccPDI is a low molecular weight protein that inhibits the growth of closely related species. Whereas the activity of some microcins extends to a range of Gram-negative bacteria including *Escherichia, Klebsiella, Salmonella*, and *Pseudomonas* (6), MccPDI has only been observed to inhibit *Escherichia*, and testing has also shown inhibition of *Shigella* but not *Salmonella* or *Klebsiella* (data not shown). In Examples presented below, the PDI$^+$ strain *E. coli*-25 was competed against a panel of pathogenic *E. coli* O157:H7 (n=25) and *E. coli* O26 (n=3). All the strains were susceptible to killing (FIG. **1**) and the average reduction following co-culture was greater than 5 logs. The degree of killing may actually be much greater as our methods to determine cell counts were limited to 2×10^3 CFU/ml. Calculations for the degree of killing were determined using 2×10^3 CFU/ml for instances where the susceptible population was undetectable. Nonetheless, PDI effectively kills greater than 99% of the competing population. Because many of these pathogens are significant in diseases of both animals and humans (33), MccPDI has application in clinical medicine, food safety and other fields. FIG. **4** shows that native mcpM transcription occurs primarily during rapid growth of the inhibitor population.

The invention provides methods and compositions for killing and/or for preventing or decreasing the adverse effects of pathogenic bacteria such as pathogenic *Escherichia coli* (*E. coli*). The methods involve contacting the pathogenic bacteria with the novel microcin described herein, microcin MccPDI, the amino acid sequence of which is presented in SEQ ID NO: 24. In some embodiments, the microcin MccPDI has undergone one or more cleavage events and comprises a sequence comprising residues 37-120 of SEQ ID NO: 24. The cleaved product may form multimers containing two, three, four, five, six, or more cleaved products as part of a disulfide-bonded complex. In exemplary embodiments, the product forms a dimer. The contact may be via a preparation of the microcin itself, or via a preparation of a bacterium encoding the microcin, as described in detail below.

A further aspect of the invention provides a composition comprising microcin MccPDI having one or more of a sequence of SEQ ID NO: 77 or a functional variant thereof and/or SEQ ID NO:24 or a functional variant thereof, wherein said functional variant has a sequence at least 95% identical to SEQ ID NO: 77 or SEQ ID NO:24; and an oxidizing agent. The composition may contain 5-30% oxidizing agent. In some embodiments, the composition contains 10% oxidizing agent or 20% oxidizing agent. An oxidizing agent is a compound that is capable removing an electron (e.g., addition of a hydrogen) from another chemical species in a redox reaction. Exemplary oxidizing agents include, but are not limited to, dimethyl sulfoxide (DMSO), sodium perborate, permanganate, hypochlorite, and hydrogen peroxide.

Hosts, Pathogens and Sources of Contamination

While most *E. coli* strains are harmless, some serotypes can cause serious and even deadly diseases in a host, either as the result of exposure to the pathogenic bacteria via direct transmission from another infected host or by ingestion of or exposure to (e.g. handling) contaminated food products or from other sources of the bacteria (e.g. fomites). In particular, the targeted pathogenic bacteria include *E. coli* strains expressing the OmpF protein, which are known to be vulnerable to the MccPDI microcin. The methods and compositions are also effective for killing (e.g. lysing) or preventing or decreasing the adverse effects of pathogenic *Shigella* sp. Those of skill in the art will recognize that phylogenetic studies indicate that *Shigella* is more appropriately treated as a subgenus of *Escherichia*, and that certain strains generally considered *E. coli* (e.g. *E. coli* O157:H7) could be classified as *Shigella*. Herein, the phrases "pathogenic bacteria" and "pathogenic *E. coli*" encompasses both pathogenic *E. coli* and pathogenic *Shigella*, although the two may be discussed separately, for clarity and to accord with historic designations.

The term "pathogenic" refers to the ability of the bacterium to cause disease symptoms in one or more hosts. The targeted bacterium need not cause disease in all hosts that is it capable of colonizing. Successful colonization of some hosts by the bacterium may be entirely benign (asymptomatic, harmless, etc.). However, such non-susceptible hosts may serve as reservoirs of the pathogenic bacteria which, when transmitted to a susceptible host, cause disease. Herein, these two genera of hosts may be referred to as "disease susceptible hosts" and "non-disease susceptible hosts", respectively, or simply as "susceptible hosts" and "non-susceptible hosts". It will be understood that the methods of treatment described herein may be advantageously applied to both susceptible and non-susceptible hosts. For the susceptible hosts, treatment may prevent, cure (fully or partially) or ameliorate disease symptoms, or prevent or decrease adverse effects that would otherwise be caused by pathogenic bacteria. These beneficial effects are brought about by killing and/or damaging established pathogenic bacteria, or by preventing, slowing or minimizing the growth of pathogenic bacteria to which the host is newly exposed. For non-susceptible hosts, treatment may destroy or lessen the number of pathogenic bacteria that can colonize the host or that might otherwise colonize the host, but for intervention using the methods and compositions described herein, thereby lessening or eliminating transmission of the pathogenic bacteria to other disease susceptible and non-susceptible hosts.

Susceptible hosts that may be subject to diseases caused by pathogenic *E. coli* are usually endotherms and may be mammals. Such mammals include but are not limited to: primates (e.g. humans), livestock e.g. cattle, pigs, sheep goats, etc., especially neonates, juveniles, elderly or immune compromised individuals; etc. Alternatively, various avian species may also be subject to such infections, including but not limited to: chickens, turkeys, ducks, etc. Non-susceptible hosts that may act as reservoirs of pathogenic bacteria that are passed to susceptible hosts include substantially the same endotherms described above as susceptible hosts.

Further, pathogenic bacteria may be transmitted among members of a particular host group (e.g. from person to person, among cows in a herd, etc.) or even from one area of an individual host organism to another area of the same organism, e.g. pathogenic bacteria may be transmitted from the anus to the urethra via fecal contamination, causing urethral infection.

Particular combinations of susceptible hosts and pathogenic bacteria include the following exemplary animal pathogens of interest:

Poultry—avian pathogenic *E. coli* (APEC)
Calves—*E. coli* K99 (which causes calf diarrhea)
Swine—*E. coli* K88 (which causes post-weaning diarrhea)
For food safety:
E. coli O157:H7
The United States Department of Agriculture (USDA) "Big 6" STEC *E. coli*
pathogens: *E. coli* serovars O26, O45, O103, O111, O121 and O145.
Diarrhoeagenic *E. coli* human pathovars:
various enteropathogenic *E. coli* (EPEC)
various enterohaemorrhagic *E. coli* (EHEC)
various enterotoxigenic *E. coli* (ETEC)
various enteroinvasive *E. coli* (EIEC; including *Shigella*)
various enteroaggregative *E. coli* (EAEC)
various so-called diffusely adherent *E. coli* (DAEC)
Extraintestinal *E. coli* (ExPEC) human pathovars:
uropathogenic *E. coli* (UPEC)
neonatal meningitis *E. coli* (NMEC)

Exemplary pathogenic *Shigella* species of interest which may be killed by the compositions and methods of the invention include but are not limited to: Serogroup A: *S. dysenteriae*, Serogroup B: *S. flexneri*, and Serogroup D: *S. sonnei*, and serotypes and serovars thereof.

In addition, contamination with pathogenic bacteria can occur via other routes of transmission such via fomites, (inanimate objects such as countertops, cutting boards, utensils, towels, money, clothing, dishes, toys, dirt, excreted feces, diapers, surfaces in barns and stockyards, etc.), or via unpasteurized milk, dairy products, juices, etc.; or via contaminated water (e.g. drinking water, ponds and lakes, swimming pools, etc.); or via contaminated animals, meat, or produce; or fruits, etc.

In some aspects, the methods of the invention involve contacting pathogenic bacteria with the microcin MccPDI. Accordingly, the invention provides i) substantially purified MccPDI microcin protein; and ii) substantially pure cultures of bacteria that produce the microcin protein.

Proteins and Nucleic Acids

In some aspects the invention provides MccPDI microcin protein and/or a gene that encodes the protein (e.g. SEQ ID NOS: 23 or 33 and 24) as well as proteins/polypeptides of the operon disclosed herein, and the genes which encode them (e.g. SEQ ID NOS: 25-32). In some embodiments, the microcin MccPDI has undergone one or more cleavage events and comprises a sequence comprising residues 37-120 of SEQ ID NO: 24 which is represented by SEQ ID NO:77. The cleaved product may form multimers containing two, three, four, five, six, or more cleaved products as part of a disulfide-bonded complex. In exemplary embodiments, the product forms a dimer.

Substantially purified MccPDI microcin protein may be produced either recombinantly, or from a native or naturally occurring source such as the bacteria described herein. Those of skill in the art are familiar with techniques for genetically engineering organisms to recombinantly produce or overproduce a protein of interest such as MccPDI. Generally, such techniques involve excision of a gene encoding the protein from a natural source e.g. using nucleases or by amplifying the gene e.g. via PCR using primers complementary to sequences that flank the gene of interest. The gene can then be inserted into and positioned within a vector (e.g. an expression vector such as a plasmid or virus) so that it is able to be expressed (transcribed into translatable mRNA). Typically, the gene that is to be transcribed is juxtaposed to one or more suitable control elements such as promoters, enhancers, etc. that drive expression of the gene. Suitable vectors include but are not limited to: plasmids, adenoviral vectors, baculovirus vectors (e.g. so-called shuttle or "bacmid" vectors, and the like). Suitable vectors may be chosen or constructed to contain appropriate regulatory sequences, including promoter sequences, terminator sequences, polyadenylation sequences, enhancer sequences, marker genes, and other sequences. The vectors may also contain a plasmid or viral backbone.

Typically, the vector is used to genetically engineer or infect a host organism where the gene is transcribed and translated into protein. In the host, the gene may be expressed from the vector (transcribed extrachromasomally, also called "in trans") and may be overexpressed, i.e. expressed at a level that is higher than normally occurs in its native bacterial host. Alternatively, the gene may be inserted into the chromosome of the host ("in cis"). Exemplary expression systems that may be utilized include but are not limited to bacteria (such as *E. coli*), yeast, baculovirus, plant, mammalian, and cell-free systems. Host bacteria may be heterologous, i.e. they may be non-native bacteria in which the gene is not present in nature. Alternatively, they may be native bacteria that are natural hosts, but which are genetically engineered to produce the microcin in greater abundance (at higher levels or concentrations) than in the native, non-engineered host. Exemplary heterologous bacterial hosts include but are not limited to: various *lactobacillus* species such as *Lactobacillus casei, Lactobacillus acidophilus, Lactobacillus fermentum, Lactobacillus gasseri, Lactobacillus pentosus, Lactobacillus plantarum, Lactobacillus sporogenes, Lactobacillus brevis, Lactobacillus delbrueckii, Lactobacillus salivarius, Lactobacillus hilgardii, Lactobacillus lactis, Lactobacillus rhamnosus, Lactobacillus johnsonii, Lactobacillus leishmanis, Lactobacillus jensenii, Lactobacillus reuteri, Lactobacillus sakei, Lactobacillus cellobiosus, Lactobacillus crispatus, Lactobacillus curvatus, Lactobacillus caucasicus*, and *Lactobacillus helveticus*, and others taught, for example, in United States patent application 20090169582 (Chua), the complete contents of which is hereby incorporated by reference in entirety; and other types of bacterial, fungal and/or viral recombinant hosts. Mammalian cells available in the art for heterologous protein expression include lymphocytic cell lines (e.g., NSO), HEK293 cells, Chinese hamster ovary (CHO) cells, COS cells, HeLa cells, baby hamster kidney cells, oocyte cells, and cells from a transgenic animal, e.g., mammary epithelial cell. For details, see Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press (1989). Many established techniques used with vectors, including the manipulation, preparation, mutagenesis sequencing, and transfection of DNA, are described in Current Protocols in Molecular Biology, Second Edition, Ausubel et al. eds., John Wiley & Sons (1992).

The vector or chromosome from which the microcin is transcribed includes at least a genetic sequence encoding the microcin described herein and may comprise one or more additional genes of the operon described herein, i.e. genes mcpM (SEQ ID NO: 23), mcpI (SEQ ID NO: 25), mcpA (SEQ ID NO: 27), mcpD (SEQ ID NO: 29), and mcpB (SEQ ID NO: 31), each of which encodes a respective protein or functional variant thereof (see below for explanation of "variant". The one or more (at least one) gene(s) in the vector or chromosome is/are expressable and are operably (functionally, expressibly) linked to one or more control or expression elements, e.g. promoters, enhancers, etc. in a manner that facilitates, causes or allows expression of the gene(s). In some aspects, the genes are present on a plasmid such as the plasmid with the nucleotide sequence shown in

SEQ ID NO: 33), or a plasmid with at least about 55, 60, 65, 70, 75, 80, 85, 90, or 95% or more (e.g. 96, 97, 98, 99%) identity. The plasmid may be located in a native host bacterium, e.g. E-25 (which is resistant to tetracycline, streptomycin and sulfa drugs) and/or E-264 (which is not antibiotic resistant).

The protein that is produced is the microcin MccPDI (or another protein encode by the operon as described above) or a physiologically active variant thereof. By "physiologically active variant" or "active variant" or "functional variant", we mean a protein sequence that is able to kill pathogenic bacteria as described herein. The protein may have the sequence shown in SEQ ID NO: 24, or may include this sequence, or a sequence that shares at least about 95% identity to SEQ ID NO: 24 (e.g. that is about 95, 96, 97, 98 or 99% identical thereto, as determined by alignment methods that are well-known), but that retains the ability to kill and/or impede growth/reproduction of and/or colonization by pathogenic bacteria. Compared to the wild type microcin, such variants are at least about 50%, and usually about 55, 60, 65, 70, 75, 80, 85, 90, or 95% or more as potent re killing, impeding growth and/or colonization, etc. In some embodiments, the variant may be more potent than the native microcin.

The variants of MccPDI that may be used in the practice of the invention may include those in which one or more amino acids are substituted by conservative or non-conservative amino acids, as is understood in the art. Further, deletions or insertions may also be tolerated without impairing the function. In addition, the microcin may be included in a chimeric or fusion protein that includes other useful sequences, e.g. tagging sequences (e.g. histidine tags), various targeting sequences (e.g. sequences that promote secretion or target the protein to a subcellular apartment or to the membrane), other antimicrobial sequences (e.g. other microcins), and the like, as well as spacer or linking sequences. The sequence of the microcin may be altered to prevent or discourage proteolysis, to promote solubility, or in any other suitable manner.

Some aspects of the invention provide a microcin with a sequence such as that shown in SEQ ID NO: 24, but which is foreshortened by 18 amino acids at the amino terminus, i.e. the 18 amino terminal residues present in SEQ ID NO: 24 are absent in this sequence, which is shown below as SEQ ID NO: 35. As described above for SEQ ID 24, active variants of the sequence represented by SEQ ID NO: 35 are also encompassed by the invention.

(SEQ ID NO: 35)

N A N S N F E G G P R N D R S S G A R N S L G R N

A P T H I Y S D P S T V K C A N A V F S G **M** I G G

A I K G G P I G **M** A R G T I G G A V V G Q C L S D

H G S G N G S G N R G S S S S C S G N N V G G T C

N R.

Some aspects of the invention provide a microcin with a sequence such as that shown in SEQ ID NO: 24, but which is foreshortened by 36 amino acids at the amino terminus, i.e. the 36 amino terminal residues present in SEQ ID NO: 24 are absent in this sequence, which is shown below as SEQ ID NO: 77. As described above for SEQ ID 24, active variants of the sequence represented by SEQ ID NO: 77 are also encompassed by the invention. The protein may have the sequence shown in SEQ ID NO: 77, or may include this sequence, or a sequence that shares at least about 95% identity to SEQ ID NO: 24 (e.g. that is about 95, 96, 97, 98 or 99% identical thereto).

(SEQ ID NO: 77)

R N S L G R N A P T H I Y S D P S T V K C A N A V

F S G **M** I G G A I K G G P I G **M** A R G T I G G A V

V G Q C L S D H G S G N G S G N R G S S S S C S G

N N V G G T C N R.

The invention also encompasses nucleic acid sequences that encode the microcin and active variants thereof as described herein. For example, the encoding sequence may be that which is represented in SEQ ID NO: 23, but this is not always the case. Variants of SEQ ID NO: 23, usually having at least about 95, 96, 97, 98, or 99% identity thereto, are also contemplated. However, those of skill in the art will recognize that the identity may be much lower (e.g. about 50, 55, 60, 65, 70, 75, 80, 85 or 90%) and the sequence may still encode a fully functional microcin, e.g. due to the redundancy of the genetic code.

Calculations of "homology" and/or "sequence identity" between two sequences may be performed as follows: The sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In a preferred embodiment, the length of a reference sequence aligned for comparison purposes is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, 90%, 100% of the length of the reference (native) sequence. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid "identity" is equivalent to amino acid or nucleic acid "homology"). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. In an exemplary embodiment, the percent identity between two amino acid sequences is determined using the Needleman and Wunsch (1970, J. Mol. Biol. 48:444-453) algorithm that has been incorporated into the GAP program in the GCG software package, using either a Blossum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In an exemplary embodiment, the percent homology/identity between two nucleotide sequences is determined using the GAP program in the GCG software package, using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. A particularly preferred set of parameters (and the one that may be used if the practitioner is uncertain about what parameters may be applied to determine if a molecule is within a sequence identity, or homology limitation of the invention) are a Blossum 62 scoring matrix with a gap penalty of 12, a gap extend penalty of 4, and a frame shift gap penalty of 5. The percent identity/homology between two amino acid or nucleotide sequences can also be determined using the algorithm of E. Meyers and W. Miller ((1988) CABIOS, 4:11-17) that has been incorporated into the ALIGN program (version 2:0); using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

The culturing and the maintenance of cultures of microorganisms such as the bacteria of the invention is carried out e.g. as described herein in the Examples section. Bacterial preparations may be lyophilized or freeze-dried.

The production of the substantially purified microcin protein is carried out by methods known to those of skill in the art, e.g. by collecting unpurified protein from a source such as the bacteria (or other expression system) that make the protein, and purifying and characterizing the protein using known steps, e.g. various separation techniques and identification techniques which include but are not limited to: centrifugation, column chromatography, affinity chromatography, electrophoresis, precipitation, sequencing, spectroscopy, etc. Preparations may be lyophilized or freeze-dried. By "substantially purified" we mean that the microcin is provided in a form that is at least about 75 wt %, preferably at least about 80 wt %, more preferably at least about 90 wt %, and most preferably at least about 95 wt % or more free from other macromolecules such as other peptides, proteins, nucleic acids, lipids, membrane fragments, etc., as is understood by those of skill in the art.

Compositions

The microcins and/or bacteria producing microcins (both of which may be referred to herein as "active agent(s) or "active ingredient(s))" of this invention will generally be used as a bactericidal active ingredient in a composition, i.e. a formulation, with at least one additional component such as a surfactant, a solid or liquid diluent, etc., which serves as a carrier. The formulation or composition ingredients are selected to be consistent with the physical properties of the active ingredient, the mode of application and environmental factors at the site of use, e.g. such as surface type, (e.g. soil or solid substrate, etc.), moisture, temperature, etc. If the composition is to be administered to a host, the ingredients are selected so as to be physiologically compatible with the host. Useful formulations include both liquid and solid compositions. Liquid compositions include solutions (including emulsifiable concentrates), suspensions, emulsions (including microemulsions and/or suspoemulsions) and the like, which optionally can be thickened into gels. The general types of aqueous liquid compositions are soluble concentrate, suspension concentrate, capsule suspension, concentrated emulsion, microemulsion and suspoemulsion. The general types of nonaqueous liquid compositions are emulsifiable concentrate, microemulsifiable concentrate, dispersible concentrate and oil dispersion.

The general types of solid compositions are dusts, powders, granules, pellets, pills, pastilles, tablets, films, filled or layered films, coatings, impregnations, gels, cakes, and the like, which can be water-dispersible ("wettable") or water-soluble. Films and coatings formed from film-forming solutions or flowable suspensions may be useful for some applications. Active ingredients can be (micro) encapsulated and further formed into a suspension or solid formulation; alternatively the entire formulation of active ingredient can be encapsulated (or "overcoated"). Encapsulation can control or delay release of the active ingredient. An emulsifiable granule combines the advantages of both an emulsifiable concentrate formulation and a dry granular formulation. High-strength compositions may be used as intermediates for further formulation.

Sprayable formulations are typically extended in a suitable medium before spraying. Liquid and solid formulations are formulated to be readily diluted in the spray medium, which may be aqueous-based, e.g. water. Spray volumes can range from about one to several thousand liters, sprayable formulations may be tank mixed with water or another suitable medium for treatment by aerial or ground application, e.g. of stockyards, barns, stables, stalls, bins containing produce, etc. Smaller volume spray formulations for use on smaller surfaces (e.g. countertops, for application to small quantities of food stuffs, etc.) are also contemplated.

The formulations will typically contain effective amounts of active ingredient in the range of about 1 to about 99 percent by weight.

Solid diluents include, for example, clays such as bentonite, montmorillonite, attapulgite and kaolin, gypsum, cellulose, titanium dioxide, zinc oxide, starch, dextrin, sugars (e.g., lactose, sucrose), silica, talc, mica, diatomaceous earth, urea, calcium carbonate, sodium carbonate and bicarbonate, and sodium sulfate. Typical solid diluents are described in Watkins et al., Handbook of Insecticide Dust Diluents and Carriers, 2nd Ed., Dorland Books, Caldwell, N.J., the complete contents of which is hereby incorporated by reference in entirety.

Liquid diluents include, for example, water, N,N-dimethylalkanamides (e.g., N,N-dimethylformamide), limonene, dimethyl sulfoxide, N-alkylpyrrolidones (e.g., N-methylpyrrolidinone), ethylene glycol, triethylene glycol, propylene glycol, dipropylene glycol, polypropylene glycol, propylene carbonate, butylene carbonate, paraffins (e.g., white mineral oils, normal paraffins, isoparaffins), alkylbenzenes, alkylnaphthalenes, glycerine, glycerol triacetate, sorbitol, aromatic hydrocarbons, dearomatized aliphatics, alkylbenzenes, alkylnaphthalenes, ketones such as cyclohexanone, 2-heptanone, isophorone and 4-hydroxy-4-methyl-2-pentanone, acetates such as isoamyl acetate, hexyl acetate, heptyl acetate, octyl acetate, nonyl acetate, tridecyl acetate and isobornyl acetate, other esters such as alkylated lactate esters, dibasic esters and .gamma.-butyrolactone, and alcohols, which can be linear, branched, saturated or unsaturated, such as methanol, ethanol, n-propanol, isopropyl alcohol, n-butanol, isobutyl alcohol, n-hexanol, 2-ethylhexanol, n-octanol, decanol, isodecyl alcohol, isooctadecanol, cetyl alcohol, lauryl alcohol, tridecyl alcohol, oleyl alcohol, cyclohexanol, tetrahydrofurfuryl alcohol, diacetone alcohol and benzyl alcohol. Liquid diluents also include glycerol esters of saturated and unsaturated fatty acids (typically C_6-C_{22}), such as plant seed and fruit oils (e.g., oils of olive, castor, linseed, sesame, corn (maize), peanut, sunflower, grapeseed, safflower, cottonseed, soybean, rapeseed, coconut and palm kernel), animal-sourced fats (e.g., beef tallow, pork tallow, lard, cod liver oil, fish oil), and mixtures thereof. Liquid diluents also include alkylated fatty acids (e.g., methylated, ethylated, butylated) wherein the fatty acids may be obtained by hydrolysis of glycerol esters from plant and animal sources, and can be purified by distillation. Typical liquid diluents are described in Marsden, Solvents Guide, 2nd Ed., Interscience, New York, 1950, the complete contents of which is hereby incorporated by reference in entirety.

The solid and liquid compositions of the present invention may include one or more surfactants. When added to a liquid, surfactants (also known as "surface-active agents") generally modify, most often reduce, the surface tension of the liquid. Depending on the nature of the hydrophilic and lipophilic groups in a surfactant molecule, surfactants can be useful as wetting agents, dispersants, emulsifiers or defoaming agents. Surfactants can be classified as nonionic, anionic or cationic. Exemplary suitable surfactants can be found, for example, in United States patent application 20130143940 to Long, the entire contents of which is hereby incorporated by reference. Also useful for the present compositions are mixtures of nonionic and anionic surfactants or mixtures of nonionic and cationic surfactants. Nonionic, anionic and cationic surfactants and their recommended uses are disclosed in a variety of published references including McCutcheon's Emulsifiers and Detergents, annual American and International Editions published by McCutcheon's Division, The Manufacturing Confectioner Publishing Co.; Sisely and Wood, Encyclopedia of Surface Active Agents, Chemical Publ. Co., Inc., New York, 1964; and A. S. Davidson and B. Milwidsky, Synthetic Detergents, Seventh Edition, John Wiley and Sons, New York, 1987, the complete contents of each of which is hereby incorporated by reference in entirety.

Compositions of this invention may also contain formulation auxiliaries and additives, known to those skilled in the art as formulation aids (some of which may be considered to also function as solid diluents, liquid diluents or surfactants). Such formulation auxiliaries and additives may control: pH (buffers), foaming during processing (antifoams such polyorganosiloxanes), sedimentation of active ingredients (suspending agents), viscosity (thixotropic thickeners), in-container microbial growth (antimicrobials), product freezing (antifreezes), color (dyes/pigment dispersions), wash-off (film formers or stickers), evaporation (evaporation retardants), and other formulation attributes. Film formers include, for example, polyvinyl acetates, polyvinyl acetate copolymers, polyvinylpyrrolidone-vinyl acetate copolymer, polyvinyl alcohols, polyvinyl alcohol copolymers and waxes. Examples of formulation auxiliaries and additives include those listed in McCutcheon's Volume 2: Functional Materials, annual International and North American editions published by McCutcheon's Division, The Manufacturing Confectioner Publishing Co., the complete contents of which is hereby incorporated by reference in entirety.

The active agents described herein and any other active ingredients are typically incorporated into the present compositions by dissolving or suspending the active ingredient in a solvent or by grinding in a liquid or dry diluent. Solutions, including emulsifiable concentrates, can be prepared by simply mixing the ingredients. The preparation may be lyophilized (freeze dried). If the solvent of a liquid composition intended for use as an emulsifiable concentrate is water-immiscible, an emulsifier is typically added to emulsify the active-containing solvent upon dilution with water. Active ingredient slurries, with particle diameters of up to 2,000 μm can be wet milled using media mills to obtain particles with average diameters below 3 μm. Aqueous slurries can be made into finished suspension concentrates (see, for example, U.S. Pat. No. 3,060,084, the complete contents of which is hereby incorporated by reference in entirety) or further processed by spray drying to form water-dispersible granules. Dry formulations usually require dry milling processes, which produce average particle diameters in the 2 to 10 μm range. Dusts and powders can be prepared by blending and usually grinding (such as with a hammer mill or fluid-energy mill). Granules and pellets can be prepared by spraying the active material upon preformed granular carriers or by agglomeration techniques. See Browning, "Agglomeration", Chemical Engineering, Dec. 4, 1967, pp 147-48, Perry's Chemical Engineer's Handbook, 4th Ed., McGraw-Hill, New York, 1963, pages 8-57 and following, and WO 91/13546. Pellets can be prepared as described in U.S. Pat. No. 4,172,714. Water-dispersible and water-soluble granules can be prepared as taught in U.S. Pat. No. 4,144,050, U.S. Pat. Nos. 3,920,442 and DE 3,246,493. Tablets can be prepared as taught in U.S. Pat. No. 5,180,587, U.S. Pat. No. 5,232,701 and U.S. Pat. No. 5,208,030. Films can be prepared as taught in GB 2,095,558 and U.S. Pat. No. 3,299,566. For further information regarding the art of formulation, see T. S. Woods, "The Formulator's Toolbox—Product Forms for Modern Agriculture" in Pesticide Chemistry and Bioscience, The Food-Environment Challenge, T. Brooks and T. R. Roberts, Eds., Proceedings of the 9th International Congress on Pesticide Chemistry, The Royal Society of Chemistry, Cambridge, 1999, pp. 120-133. See also U.S. Pat. No. 3,235,361, Col. 6, line 16 through Col. 7, line 19 and Examples 10-41; U.S. Pat. No. 3,309,192, Col. 5, line 43 through Col. 7, line 62 and Examples 8, 12, 15, 39, 41, 52, 53, 58, 132, 138-140, 162-164, 166, 167 and 169-182; U.S. Pat. No. 2,891,855, Col. 3, line 66 through Col. 5, line 17 and Examples 1-4; Klingman, Weed Control as a Science, John Wiley and Sons, Inc., New York, 1961, pp 81-96; Hance et al., Weed Control Handbook, 8th Ed., Blackwell Scientific Publications, Oxford, 1989; and Developments in formulation technology, PJB Publications, Richmond, U K, 2000. The complete contents of each of these references is hereby incorporated by reference in entirety.

In addition, the formulations may include other suitable active agents, e.g. other antimicrobial agents such as other microcins, antibiotics, etc.; or broadly defined antimicrobials such as antiseptics or heavy metals, etc.

Incorporation into Various Products

The active agents described herein may be incorporated into and/or used as an amendment to many different products, e.g. substrates and media which include but are not limited to: so-called "hand-sanitizing" preparations and soaps, gels, etc.; various sprays and washes; detergents and various cleaning agents; fabrics e.g. linings for materials such as diapers and other garments that may be contacted by feces; "booties" that are used to cover and protect shoes; disposable or non-disposable gloves; disposable or non-disposable food preparation surfaces, e.g. as sheets of material that can be placed on a cutting surface, or in a cutting surface itself; in storage apparatuses for implements used in food preparation (e.g. knife blocks, or holders, etc.); and others.

In some aspects, the active agents described herein are incorporated into packaging materials, e.g. packaging materials designed to contain meat or meat products or produce. For example, the packaging material may be impregnated with the active agent either during or after manufacture, or may be coated onto one or more surfaces of the material. The packaging material may be a film e.g. formed from a flexible polymer that may be transparent, or may be a rigid or semi-rigid container formed from e.g. plastic resin, styrofoam, wood, cardboard or pasteboard or other molded cellulose product, or made from some other so-called "natural" material. The packaging material may be in the form of "peanuts". The material may be biodegradable. United States patent applications 20120259295 (Bonutti) and 20030234466 (Rasmussen) and references cited therein, the complete contents of all of which are hereby incorporated by reference in entirety, discuss the preparation of various types of packaging materials.

The active agents may be incorporated into probiotic formulations. Such formulations may be designed or tailored to suit the mode of administration and the host to which the probiotic is administered. For example, if the targeted host is a human, the active agents may be added to other known probiotic products (kefir, yogurts, "smoothies", etc.) and/or other ingredients that increase palatability may be added (e.g. flavorings, thickeners, coloring agents, etc.). The formulation may be chewable (e.g. a gum or tablet) or taken as a pill. Other organisms may also be present in the probiotic preparation e.g. lactic acid bacteria (LAB), bifidobacteria, yeasts and various bacilli. If the recipient host is a juvenile such as a calf, the probiotic may be a milk substitute formulation. If the recipient is a bird or fowl, the probiotic may be a formulation of drinking water. Probiotics may also be formulated as suppositories.

Methods and Uses

In some aspects, the invention provides methods of using the microcins and bacteria that produce the microcins described herein, for preventing or decreasing the transmission of pathogenic *Escherichia coli* (*E. coli*) bacteria from a first location to a second location, e.g. from a first host (that may or not be a susceptible host) or first contaminated area, to a second host or previously uncontaminated area. The second host may or may not be susceptible. The first location may be a "reservoir" host or area/location that is already colonized by the pathogenic bacteria. Alternatively, the first host or location may be likely to be colonized or possible to colonize.

Administration to Hosts

If the first location is a susceptible (or non-susceptible) first host, the method comprises administering to the first host the microcin described herein or a bacterium that contains and expresses a nucleotide sequence encoding the microcin. By "administering" we mean the deliberate, intentional, active introduction of the bacterium into the first host (i.e. the purposeful inoculation of the first host), usually by a human or by a device, instrument or machine designed and operated by a human. In other words, the bacterium is not inadvertently, passively or accidentally transmitted, or is not transmitted as the result of an act of nature, or as the result of contamination of a source of the bacteria. Generally, the "bacterium" that is deliberately administered is a substantially pure, genetically homogenous population of substantially identical bacteria, or part of a mixture of several types of such substantially pure bacteria (e.g. several different serotypes, serovars, or strains. The bacteria that are so administered are generally cultured in vitro for a time prior to administration, and the method may involve culturing the bacteria from a natural source, selecting a single colony for propagation, and propagating the bacteria to form a culture that is sufficiently large or populous to successfully inoculate a host.

Administration results in contact between pathogenic bacteria that reside in/on the first host and the killing or damaging, etc. of the pathogens. Alternatively, administration may be prophylactic, i.e. the first host is not already infected with the pathogen, and infection is prevented or decreased. If bacteria are administered, the step of administering may also result in colonization of a host that is treated with the administered bacteria, i.e. bacteria that have the gene encoding the microcin. Thus, in some aspects, the step of administering results in an alteration of the microflora (e.g. "gut" or "digestive tract" microflora) of the recipient host, and the bacteria thus are a "probiotic" as discussed elsewhere herein, competing for nutrition and attachment sites to within the host. "Digestive tract" includes e.g. the mouth, esophagus, stomach, small intestine and large intestine (which includes the cecum, colon and rectum). In general, the amount of microcin that is administered in order to be effective is in the range of from about the amount of microcin would range between about 1 ug and 100 mg depending on the application and dilution factor; and the amount of bacteria that is administered in order to be effective is in the range of from about 10^3 to about 10^{12}, and is preferably in the range of from about 10^6 to about 10^9. Those of skill in the art will recognize that variations may occur, depending e.g. on how much microcin is produced by the bacterial strain in question, by the species, size, age, etc. of the subject to whom the microcin and/or the bacteria (or other recombinant host that produces the microcin) is administered.

When the microcin is administered, it may be in any suitable form or incorporated into any suitable vehicle. Exemplary vehicles for administering the microcin include but are not limited to: liquids such as drinking water, formula, and the like; and solid or semi-solid forms such as suppositories, pills, tablets, etc. The vehicle may be a solid "slow release" vehicle. The vehicle may include or be contained within e.g. a permeable or semi-permeable bag or pouch which can be suspended or retained indefinitely in the gut of a host organism (e.g. a cow), from which the active agent leaches or is released over time. The bag or pouch may be biodegradable.

For avian hosts (e.g. chickens), an exemplary mode of administration is addition of microcin-producing bacteria to drinking water or feed. Administration in this manner may be termed "probiotic" because the goal is to encourage colonization of the bird's digestive system with the harmless, protective bacteria, although colonization is not a requirement for positive effects to accrue. The protective microcin-producing bacteria can destroy or kill and thus outcompete pathogenic bacteria encountered by the bird, preventing colonization by the pathogens, or a least decreasing the level of colonization of, and hence transmission from, the bird. If the microcin itself is added to the drinking water or feed, it will destroy or kill pathogenic bacteria encountered by the bird, preventing colonization by the pathogens, or a least decreasing the level of colonization of, and hence transmission from, the bird, and possibly allow other non-pathogenic bacteria to flourish.

Similar strategies may be employed for bovine hosts, e.g. addition of microcin-producing bacteria or the microcin itself to drinking water, feed, salt licks, calf formula, etc., or administration of the bacteria as a probiotic to encourage the establishment of microcin-producing bacteria as described herein, or to provide a protective shield against infection by pathogenic bacteria.

Application to Surfaces

Those of skill in the art will recognize that it is also beneficial to prevent (discourage, impede, lessen, decrease, etc.) transmission of pathogenic bacteria from non-host sources to possible hosts, e.g. to prevent transmission from surfaces or areas which harbor the pathogens. The invention also comprises methods of doing so by applying the microcin of the invention and/or bacteria encoding the microcin, to surfaces which harbor the pathogens, or which are suspected or harboring the pathogens, or which could become contaminated with pathogens. Applying or treating such surfaces may be accomplished by any of many methods, e.g. by spraying a preparation of the microcin or bacteria, by applying a composition comprising a powder or granules, etc. Suitable compositions are described above. In general, the amount of microcin that is applied to a surface in order to be effective is in the range of from between about 1 ug and 100 mg; and the amount of bacteria that is applied is in the range of from about 10^3 to about 10^{12}, and is preferably in the range of from about 10^6 to about 10^9.

Areas that are particularly prone to contamination with pathogenic bacteria include those which house of livestock or fowl. Such areas, especially commercial areas, may be treated using the compositions of the invention, especially spray formulations. The areas may or may not be associated with a commercial enterprise, e.g. they may be associated with for profit or non-profit farms, stables, etc. The areas may also be set aside for animals e.g. as reserves, zoos, stockyards etc., or may be located at veterinary facilities. The compositions of the invention may be applied to any suitable surface where the microcin may be useful to kill pathogenic bacteria, e.g. soil or grass, flooring, stalls, pens, milking carousels, feed lot surfaces, drinking and/or feeding containers, cages, crates, truck beds, etc. Exemplary animals which are housed in such areas and are potential hosts of pathogenic bacteria include but are not limited to: livestock e.g. horses, mares, mules, jacks, jennies, colts, cows, calves, yearlings, bulls, oxen, sheep, goats, lambs, kids, hogs, shoats, pigs, bison, and others; and avian species such as land and water fowl e.g. chickens, turkeys, ducks, geese, ostriches, guinea fowl, etc. The preparations of the invention may be applied to the animals themselves, or to specific areas of the animals, e.g. to feet, the anal area, etc.

In addition, the preparations of the invention may be applied to various products, especially products derived from animals that are susceptible to infection with and/or to disease caused by pathogenic bacteria. The preparations may be applied to or included in (mixed into), for example, meats or meat products (including both raw and so-called "ready to eat" meat and poultry products), eggs, hides, carcasses, horns, hooves, feathers, etc.

Diseases Prevented or Treated

The types of diseases and conditions that may be prevented or treated using the methods and compositions disclosed herein include any of those which are caused by pathogenic *E. coli*, including but are not limited to: food poisoning (e.g. in humans), gastroenteritis, diarrhea, urinary tract infections, neonatal meningitis, hemolytic-uremic syndrome, peritonitis, mastitis, septicemia and Gram-negative pneumonia, shigellosis, dysentery, etc. In some aspects, probiotic preparations are contemplated, e.g. liquid or solid preparations that are taken prophylactically to prevent or treat disease symptoms or so-called Traveler's diarrhea prior to or during travel.

Herein, where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present invention, representative illustrative methods and materials are now described.

All publications and patents cited in this specification are herein incorporated by reference as if each individual publication or patent were specifically and individually indicated to be incorporated by reference and are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. The citation of any publication is for its disclosure prior to the filing date and should not be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

It is noted that, as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as "solely," "only" and the like in connection with the recitation of claim elements, or use of a "negative" limitation.

As will be apparent to those of skill in the art upon reading this disclosure, each of the individual embodiments described and illustrated herein has discrete components and features which may be readily separated from or combined with the features of any of the other several embodiments without departing from the scope or spirit of the present invention. Any recited method can be carried out in the order of events recited or in any other order which is logically possible.

Before exemplary embodiments of the present invention are described in greater detail, it is to be understood that this invention is not limited to particular embodiments described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

EXAMPLES

Example 1. Materials and Methods

Bacterial Strains, Media, and Culture Conditions.

E. coli strains (Table 1) were cultured in Luria-Bertani (LB) media (Fisher Scientific, Pittsburgh, Pa.) or in M9 minimal media (6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 1 g/l NH_4Cl, 2 mg/L thiamine, 1 mM $MgSO_4$ 0.1 mM $CaCl_2$ and 0.2% glucose) at 37° C. with shaking (200 rpm), unless stated otherwise. Components for the M9 media were purchased from Fisher Scientific (Pittsburgh, Pa.), Sigma-Aldrich (St. Louis, Mo.) and J.T. Baker Reagents and Chemicals (Phillipsburg, N.J.). Antibiotics were added to media at the following concentrations: ampicillin (amp) 100 μg/ml (Fisher Biotech, Fair Lawn, N.J.); kanamycin (kan) 50 μg/ml (Fisher Scientific, Pittsburgh, Pa.); nalidixic acid (nal) 30 μg/ml (MP Biomedicals, Solon, Ohio); and tetracycline (tet) 50 μg/ml (Fisher Scientific, Pittsburgh, Pa.). Strains that would otherwise be antibiotic susceptible were selected for nalidixic acid resistance through successive passage in LB media with increasing nalidixic acid concentrations until the strains were capable of growth at 30 μg/ml.

TABLE 1

E. coli strains and PCR primer sequences used in this work.

Strain	Genotype/ phenotype	Primers: Homologous extensions (H1^a and H2^b); PDI, rpoD, and mcpM loci	Ref.
E. coli-25	Wild-type, SSuT^R, PDI$^+$	PDI fwd: TAGTTGCAGGGGCATAAGAA (SEQ ID NO: 1)	(17)
		PDI rev: AGGAAACGCAAACAGCAACT (SEQ ID NO: 2)	
		rpoD fwd: CAGGTTCAATGCTCCGTTGC (SEQ ID NO: 3)	
		rpoD rev: GCGACCTTTCGCTTTGATGG SEQ ID NO: 4)	
		mcpM fwd: CCGTAATGACCGTTCCAGT (SEQ ID NO: 5)	
		mcpM rev: CCATTTCCACTACCATGATCT (SEQ ID NO: 6)	
E. coli-25ΔtolC	SSuT^R, Kan^R, ΔtolC, PDI$^-$	H1: ATAACCCGTATCTTTACGTTGCCT TACGTTCA (SEQ ID NO: 7)	This work
		H2: CTAGAATCCGCAATAATTTTACAGTTTGAT (SEQ ID NO: 8)	
E. coli-25ΔtraM	SSuT^R, Kan^R, ΔtraM, PDI$^+$	H1: AATAACGTGATTGCATATTACTTATCTCAGGAGTTC (SEQ ID NO: 9)	This work
		H2: ATCCCTGGAAGGACTACAACCTATGACCGAAAATAC (SEQ ID NO: 10)	
E. coli-25ΔmcpM	SSuT^R, Kan^R, ΔmcpM, PDI$^-$	H1: GTAATTTAATAAACATAGTAGCGCCCTCCATTATATCTAT (SEQ ID NO: 11)	This work
		H2: AACGCACAAAATAACAAACAACCGATAGGGGAAATATGAT (SEQ ID NO: 12)	
E. coli-25ΔmcpMΔmcpI	SSuT^R, Kan^R, ΔmcpMΔmcpI, PDI$^-$	H1: ATTATCTTTACTATATTTATATATGTTATCATTCATAATG (SEQ ID NO: 13)	This work
		H2: AACGCACAAAATAACAAACAACCGATAGGGGAAATATGAT (SEQ ID NO: 14)	
E. *coli*-25ΔmcpMΔmcpI+ pMcpI	SSuT^R, Kan^R, ΔmcpMΔmcpI, PDI$^-$, immune to PDI	H1: TGGTGATGAATTCCTGTCAAA (SEQ ID NO: 15)	
E. coli-25ΔmcpB	SSuT^R, Kan^R, ΔmcpB, PDI$^-$	H2: TACCAGTTTCACCCGTCACA (SEQ ID NO: 16)	This work
		H1: TCAGCCATTCCCATAAATGACGAGTATCAAGGTTGACG (SEQ ID NO: 17)	
E. coli-25ΔmcpD	SSuT^R, Kan^R, ΔmcpD, PDI$^-$	H2: TTGACGGAAAGGTTACTTATTGTATTAAAAATAATG (SEQ ID NO: 18)	This work
		H1: GATATACATCTGACCTGTGTGATGTTAAAGTTTTATACTA (SEQ ID NO: 19)	
E. coli-25ΔmcpA	SSuT^R, Kan^R, ΔmcpA, PDI$^-$	H2: ATAGAAAAAATAAGAACAATCTCCGCGAAATAGCATTATG (SEQ ID NO: 20)	This work
E. coli-4	Wild-type, SSuT^R, PDI$^-$		(32)
E. coli-4pPDI	SSuT^R, Kan^R, pPDI, PDI$^+$		This work
E. coli-6	Wild-type, SSuT^R, PDI$^-$		(32)
E. coli-82	Wild-type, SSuT^R, PDI$^-$		This work
E. coli-186	Wild-type, Nal^R, PDI$^-$		(32)
E. coli-264	Wild-type, Nal^R, PDI$^+$		(32)
O157: H7 Sakai	Wild-type		(15)
E. coli K12	Nal^R		(19)

[a] *E.* coli-25 gene-specific sequences are shown. For gene deletion mutants, homologous extensions also had the kanamycin primer site: TGTGTAGGCTGGAGCTGCTTCG (SEQ ID NO: 21), 3' to the *E. coli*-25 specific sequence.

[b] *E. coli*-25 gene-specific sequences are shown. For gene deletion mutants, homologous extensions also had the kanamycin primer site: CATATGAATATCCTCCTTA (SEQ ID NO: 22), 3' to the *E. coli*-25 specific sequence.

Competition Assays to Measure Inhibition Phenotype.

Bacterial strains were grown individually overnight in LB. Equal volumes of each competing strain were inoculated into fresh M9 media at a 1:200 dilution, for a final 1:100 dilution of total cells. Cultures were then incubated at 37° C. for 8 to 24 h. It was previously shown that the PDI phenotype does not differ significantly between 8 and 24 h competitions (32). Mixed cultures were then serially diluted, plated on LB supplemented with the appropriate antibiotic to select for each competing strain, and enumerated.

Live/Dead Staining and Flow Cytometry.

Viability assays were conducted using the Live/Dead® BacLight™ Bacterial Viability Kit (L34856, Molecular Probes, Invitrogen, Eugene, Oreg.). Cell cultures were grown in M9 media for six hours at 37° C. and then 1 ml of each culture was collected by centrifugation, washed in 0.85% NaCl, and resuspended in 0.85% NaCl. Cells were then diluted 1:10 in 0.85% NaCl that contained 1.5 μl of 3.34 mM SYTO 9 and 1.5 μl of 30 mM propidium iodide. Samples were incubated at room temperature in the dark for 15 min. Flow cytometry was performed on a FACCalibur flow cytometer (BD Biosciences) and data was analyzed using FCS Express software (De Novo software, Thornton, Ontario, Canada). Initial parameters were established by analyzing cell suspensions with known live- and dead-cell populations. These bacterial suspensions were prepared as follows: cells were grown in M9 minimal media to late-log phase and 1 ml aliquots of the cultures were collected by centrifugation, washed in 0.85% NaCl, and resuspended in either 0.85% NaCl (live portion) or 70% isopropyl alcohol (dead portion). Samples were incubated at room temperature for 30 min, then processed and analyzed by flow cytometry as described above. Ratios of live to dead cells used for the standard were (live:dead): 0:100, 50:50, and 100:0. Gates specific to our *E. coli* (based on side and forward light scatter) were used to collect data on 50,000 cell events. Green versus red fluorescence was measured to distinguish between SYTO 9 stained live cells and propidium iodide-SYTO 9 stained dead cells. Nonspecific signal was excluded at the time of data acquisition.

Sequencing and Analysis.

Genomic extractions of *E. coli*-25, *E. coli*-82, and *E. coli*-264 were prepared using the DNeasy Blood & Tissue kit (Qiagen, Valencia, Calif.) according to the manufacturer's instructions. Sequencing was conducted at the Genomics Core Lab at Washington State University using a Roche 454 FLX Titanium Genome Sequencer to a depth of 24× represented by 399,076 reads. Sequences were assembled using Newbler (version 2.5.3). Annotation employed Glimmer version 3.02 for gene calling, and then the data was piped into CLC Genomics Workbench (CLC Bio, Cambridge, Mass.) where the resulting genes were screened against the current BLAST, SignalP and Pfam databases for functional predictions. The annotated sequence has been deposited in GenBank under (note: sequence submitted, accession number pending).

Site-Directed Gene Deletion.

Gene-specific knockouts were generated using the methods described by Datsenko and Wanner (5). Briefly, the gene of interest was replaced with a PCR-generated kanamycin resistance marker. PCR primers were designed to amplify the kanamycin resistance gene from the template plasmid pKD4. Each primer incorporated 36-50 nt of the region flanking the gene of interest (Table 1). PCR products were column purified (Qiagen, Valencia, Calif.), digested overnight at 37° C. with DpnI (New England Biolabs, Ipswich, Mass.), purified again, and suspended in 30 μl 10 mM Tris, pH 8.0. *E. coli*-25 carrying the λ Red plasmid pKD46 (Amp^R) were grown in SOB (2% bactotryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$) with 1 mM L-arabinose at 30° C. to an OD_{600} of ~0.6. The cells were then made electrocompetent by washing twice with ice-cold water, once with 10% glycerol, and concentrating the cells 100-fold in 10% glycerol. Electrocompetent cells (50 μl) were pulsed with ~100 ng of PCR product using the Gene Pulsar 1 (Bio-Rad, Hercules, Calif.). SOC media (12) was immediately added to the cells that were then incubated 2 h at 30° C. Cells were plated on LB with kanamycin and incubated overnight at 30° C. to select for transformants. PCR amplification using primers within the kanamycin resistance gene combined with genomic primers adjacent to the sequence of interest were used to verify that the resistance cassette integrated at the desired location.

Complementation of mcpI Knockout.

A pET100 TOPO® vector (Invitrogen, Grand Island, N.Y.) was used for inducible expression of the putative immunity gene. This gene was PCR amplified from *E. coli*-25 using primers that produce a 3' single-stranded overhang identical to the 5' end of the pET100 vector, allowing directional joining of our gene of interest and the vector. Ligation and transformation was conducted according to the instructions of the Champion™ pET Directional TOPO® Expression kit. Briefly, 2 μl of PCR product was added to 1 μl Salt Solution (provided in kit), 1 μl TOPO vector, and 2 sterile water. This reaction incubated at room temperature for 25 min and then was placed on ice for 30 min. An aliquot (3 μl) was added into 50 μl chemically competent Top10 *E. coli* and incubated on ice for 2 min. Cells were heat shocked for 30 sec at 42° C. then transferred to ice. SOC media (250 μl) was added and the cells were incubated 1 h at 37° C. Cells were then plated on LB agar containing ampicillin and incubated overnight at 37° C. Transformants were screened by PCR using the universal T7 forward and reverse primers to identify clones containing the pET100 vector with an insert. Five transformants were selected for sequencing to verify they contained the correct insert. Plasmid was then isolated using the PureYield™ Plasmid Miniprep System (Promega, Madison, Wis.) and transferred to a host cell by electroporation as described above. Transformants were selected by their growth on LB with ampicillin.

Transforming *E. coli*-4 with the PDI Plasmid.

The pPDIΔtraM plasmid was purified using the MiniPrep Express™ Matrix (MP Biomedicals, Solon, Ohio). *E. coli*-4 was then made electrocompetent and transformed (as described above for the gene deletion mutants) with pPDIΔtraM. Successful transformants were selected on LB with kanamycin and PCR verified for the presence of the PDI region.

Plasmid Mating Experiments.

E. coli-25ΔmcpM and *E. coli* K12 were grown overnight in LB media with kanamycin or nalidixic acid, respectively. Equal amounts of plasmid-bearing strain *E. coli*-25ΔmcpM were mixed with non-plasmid-bearing *E. coli* K12 and centrifuged for 3 min at 16,000×g. The cells were washed and concentrated 100-fold in 10 mM $MgSO_4$. Cell suspensions were then pipetted onto a nitrocellulose membrane placed on a non-selective LB-agar plate. Following 24 h incubation at 30° C., the cells were resuspended in sterile PBS and dilutions plated on LB-agar containing nalidixic acid and/or kanamycin. The conjugation efficiency was calculated by dividing the CFU of transconjugants by the CFU of donor cells. Plasmid profiles were prepared for a subset of transconjugants to confirm the presence of plasmid. Profiles were conducted as described by Kado and Liu (14). The same experiments, using kanamycin and tetracycline for selection, were then repeated using the K12 pPDIΔmcpM transconjugant and *E. coli*-6 to determine whether the plasmid is self-mobilizable.

RNA Isolation, First-Strand cDNA Synthesis, and Microcin RT-qPCR.

E. coli-25 encoding McpM was inoculated into 5 ml M9 minimal media containing tetracycline and incubated overnight at 37° C. One ml of overnight culture was inoculated into 300 ml of room temperature M9 media and incubated at 37° C. Aliquots containing approximately 10^8-10^9 cells were removed immediately after inoculation (0 h), and 2 h, 4 h, 6 h, 8 h, 10 h, 12 h, and 24 h post-inoculation. Cells were pelleted by centrifugation at 4° C. and total RNA was isolated and DNase treated using RiboPure-bacteria kit (Ambion) according to manufacturer instructions. RNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer. RNA samples from a given time course experiment were diluted to the same concentration as the least concentrated sample. To assess DNA contamination in RNA samples prior to cDNA synthesis and expression analysis, equivalent RNA concentrations to be used in the corresponding cDNA RT-qPCR reactions were run under identical conditions used for RT-qPCR analysis using polymerase sigma subunit rpoD primers (Table 1). RNA samples with cycle threshold (Ct) values less than 37.5 cycles were again treated with DNase and Ct values reanalyzed prior to cDNA synthesis. First-strand cDNA synthesis was completed using 8 μl of RNA (2-20 ng/μl), random hexamers and SuperScript III reverse transcriptase (Invitrogen) in a final reaction volume of 20 μl according to manufacturer instructions. To verify the specificity of the mcpM primers (Table 1), a single PCR product of the correct size (213 bp) was detected in PDI$^+$ strains but not in PDI$^-$ strains when analyzed on agarose gels. rpoD primers, described above, amplified a single PCR product of the correct size (336 bp) when analyzed on agarose gels from all PDI$^+$ and PDI$^-$ *E. coli* strains tested. The amplification efficiency of primer sets was then determined using plasmid DNA encoding their respective targets under identical conditions used for RT-qPCR.

All RT-qPCR reactions were performed as a single-plex reaction in triplicate in 96-well plates. Positive controls and no template controls were included in duplicate for each primer set. Each reaction was performed using 2 μl of cDNA, 500 nM final concentration per primer, and SsoFastEva Green Supermix (Bio-Rad) in a final volume of 20 μl. All PCR reactions were performed on a CFX96 Real-Time PCR Detection System with version 2.1 software (Bio-Rad) with the following cycling conditions: 95° C. for 30 s, 40 cycles of 95° C. for 1 s, 55° C. for 5 s, and 72° C. for 15 s. Normalized (ΔΔCt) microcin expression was automatically computed using the Bio-Rad CFX Manager Software version 2.1 using rpoD as the reference gene.

Example 2. PDI is Effective Against a Broad Range of *E. coli*

In this Example, PDI was shown to be effective against a broad range of *E. coli*. Because *E. coli* O157:H7 is represented by a diversity of genetic types (33) we first determined if the PDI phenotype was effective against the representative panel of strains. Strains representing bovine-biased and clinical-biased genotypes (33) from both the U.S. and New Zealand were highly susceptible to the PDI phenotype with an average reduction >5 log compared to the population for their respective monocultures (FIG. **1**). Three strains of *E. coli* O26 were also tested, and similar reductions were found in population numbers (FIG. **1**).

Example 3. Live/Dead Staining Indicated that PDI is Bactericidal

In this example, Live/dead staining indicated that PDI is bactericidal. Although susceptible cells show a substantial reduction in their CFU/ml following competition with PDI$^+$ strains, it was not clear if the effect is bacteriostatic or bactericidal. Live/dead staining was used in conjunction with flow-cytometry to address this question using *E. coli*-25 and *E. coli* O157:H7 Sakai in mono- or co-culture. The percent of dead cells detected from the two mono-cultures was 0.50±0.06% and 0.11±0.01%, respectively (mean±SEM). When co-cultured for six hours the percent of dead cells increased to 1.27±0.09% consistent with killing of *E. coli* O157:H7 Sakai. When this susceptible strain was co-cultured with a PDI$^-$ strain (*E. coli*-6), the percentage of dead cells was 0.33±0.03%. These results, which were based on 3 independently replicated assays, indicate that PDI functions by killing susceptible cells.

Example 4. The Microcin-Encoding Gene Cluster was Identified

In this example, the microcin-encoding gene cluster was identified. In addition to the two PDI$^+$ strains, *E. coli*-82 was identified from earlier work (16) as genetically similar (no differences) to *E. coli*-25 based on Xba-I macro-restriction, pulsed-field gel electrophoresis (PFGE) profile (unpublished data). Despite having a comparable genetic profile, *E. coli*-82 does not express the PDI phenotype. Genome sequencing of these two strains allowed an in-depth comparison that identified one relatively large region of sequence difference that was located on a large plasmid in *E. coli*-25. Although previous work using a different method did not detect the presence of plasmids in *E. coli*-25 (32), these results were verified by plasmid purification (14) and subsequent Southern analysis probing for mcpA (data not shown). PCR amplification of the PDI locus (primers available in Table 1) confirmed this region is present in the PDI$^+$ strains *E. coli*-25 and *E. coli*-264, but not the PDI$^-$ strains *E. coli*-6, *E. coli*-82, or *E. coli*-186.

The *E. coli*-25 IncI1 microcin-containing plasmid is 98,809 bp with a G+C content of 49% and a coding density of 88%. Annotation of the 132 coding sequences revealed that most of the plasmid content is devoted to genes involved in transfer, including a Ira system and a pil system, or encodes proteins of unknown function. The novel region of interest is a locus of approximately 4,800 bp that encodes five genes (FIG. **2**). Two genes, which we have designated mcpB and mcpD, encode homologs of HlyB and HlyD that are known to be the structural components of a microcin transfer system along with chromosomally encoded tolC (7, 10, 18, 26). McpA, which contains a CaaX protease domain (PF02517), is thought to be the "activity" protein that processes the microcin, encoded by mcpM, to its mature form prior to transfer from the cell. mcpI likely encodes an immunity protein. This novel sequence was also found in a recent GenBank submission of *E. coli* DEC10F (Accession AIGU01000076; version: AIGU01000076.1 GI:378122919; incorporated by reference herein in its entirety).

Example 5. Knockout Mutations from *E. coli*-25 Blocked PDI

This example shows that knockout mutations from *E. coli*-25 blocked PDI. Four of the five putative microcin genes (FIG. **2**) were individually knocked out in *E. coli*-25 to analyze their role in PDI. The mcpI gene knockout was constructed as a double knockout, ΔmcpM ΔmcpI. Each region of interest was replaced with a kanamycin resistant cassette and verified by PCR for the correct insertion site. Subsequently each knockout mutant was put into competition with the PDI$^-$ *E. coli*-186 to determine whether the deletion affected the PDI phenotype. CFU counts following co-culture in M9 minimal media showed that *E. coli*-186 was greatly inhibited by *E. coli*-25 but was no longer inhibited by the ΔmcpD, ΔmcpB, ΔmcpM, ΔmcpMΔmcpI, and ΔmcpA mutants (FIG. **3**A). Conversely, each mutant was also competed with *E. coli*-264 to determine how each knockout affected immunity to PDI (FIG. **3**B). Only the ΔmcpM and ΔmcpMΔmcpI strains became susceptible to PDI indicating that the other knockout strains retained immunity. Immunity was fully restored when mcpI was complemented back into the ΔmcpMΔmcpI strain, verifying that this gene is required for resistance to killing by PDI$^+$ strains (FIG. **3**B). Expression of mcpI in a PDI$^-$ strain does not confer immunity, indicating this gene alone is insufficient to prevent inhibition from the microcin (data not shown).

Class II microcins are typically secreted by a T1SS and the presence of the putative ABC transporter and membrane-fusion genes, mcpB and mcpD, is consistent with this structure in *E. coli*-25. These secretion systems require co-expression of a chromosomally encoded TolC protein on the cell surface (7, 10, 18, 26). Consequently, a ΔtolC strain was constructed and this disrupted the ability of *E. coli*-25 to inhibit *E. coli*-186 (FIG. **3**A) but it did not influence immunity (FIG. **3**B). These results are consistent with the requirement for a T1SS for PDI function. To verify that the gene knockout procedure was not producing artifacts, a gene deletion in an unrelated region of the plasmid was also generated. As expected, deleting traM did not affect inhibition or immunity (FIG. **3**).

Example 6. Transferring the PDI Plasmid to a Non-Inhibitor *E. coli* Conferred the Inhibitory Phenotype

This example shows that transferring the PDI plasmid to a non-inhibitor *E. coli* conferred the inhibitory phenotype. Although the above knockout mutants verified that the genes involved with PDI had been identified, it was desirable to confirm from the mutants that all the genes unique to PDI were included on pPDI. Generating the traM mutant in *E. coli*-25 provided a selectable marker on pPDI that did not interfere with the PDI phenotype (see above). Following transformation with pPDIΔtraM, *E. coli*-4 acquired the ability to inhibit susceptible strains, and immunity to inhibition by *E. coli*-264 (Table 2).

Table 2 shows competition results of wild-type *E. coli*-4 and *E. coli*-4 carrying the PDI plasmid verifies the PDI genes are present on the plasmid. CFUs of PDI$^-$ *E. coli*-186 following co-culture with wild-type *E. coli*-4 or *E. coli*-4+pPDI verifies the plasmid confers the inhibitory phenotype. Immunity to PDI is also maintained on the plasmid, as indicated by the ability of *E. coli*-4+pPDI to survive co-culture with the PDI$^+$ strain *E. coli*-264. Results are expressed as log CFUs/ml±the SEM of 3 replicates.

This data indicates that all the PDI-specific genes are present on pPDI. Nevertheless, there is a possibility that other chromosomally-encoded genes common to *E. coli*-25 and *E. coli*-264 are involved with PDI expression or function.

TABLE 2

Competition results of wild-type *E. coli*-4 and *E. coli*-4 carrying the PDI plasmid verified the PDI genes are present on the plasmid.

	Log_{10}(CFU)/ml	
Competition	*E. coli*-4	Competitor
E. coli-4 (PDI$^-$)		
vs. *E. coli*-186 (PDI$^-$)	8.83 ± 0.05	8.64 ± 0.06
vs. *E. coli*-264 (PDI$^+$)	4.70 ± 0.28	9.14 ± 0.02
E. coli-4 + pPDI		
vs. *E. coli*-186 (PDI$^-$)	9.18 ± 0.06	4.03 ± 0.61
vs. *E. coli*-264 (PDI$^+$)	8.88 ± 0.03	8.76 ± 0.02

Example 7. The PDI ΔmcpM Plasmid was Shown to be Self-Transmissible

This example shows that the PDI ΔmcpM plasmid is self-transmissible. Filter mating experiments between the ΔmcpM mutant and *E. coli* K12 showed the PDI plasmid is mobile with a conjugation efficiency ranging between 4.81×10^{-6} and 3.66×10^{-6}. Plasmid profiles of K12 Kan^R verified the presence of a single plasmid of ~100 Kb, consistent with the PDI plasmid. Another series of conjugation experiments between the K12 transformants and *E. coli*-6 confirmed that the plasmid is self-transmissible. Using this assay conjugation of the PDI plasmid was not detected when there was a functional microcin system, presumably because recipient cells are killed.

Example 8. The Kinetics of the Expression of mcpM were Determined

This example shows the kinetics of the expression of mcpM. Expression of the PDI phenotype has been observed throughout log-phase growth, but this function appears to subside when cells enter stationary phase (32). This earlier work was limited by the analytic sensitivity of the phenotypic assay and thus to better describe the kinetics of microcin expression we employed a quantitative PCR assay. RT-qPCR data confirmed that expression of the microcin increases rapidly during the log-phase growth and drops off rapidly in stationary phase (FIG. **4**).

Example 9. cDNA Sequences and Protein Sequences for mcpM, mcpI, mcpA, mcpD, and mcpB were Determined

This example shows the cDNA sequences and protein sequences encoded by mcpM, mcpI, mcpA, mcpD, and mcpB:

```
cDNA sequence and protein sequence for mcpM
                              (SEQ ID NOS: 23 and 24)
ATGGCAAATATAAGAGAATTAACTTTAGATGAGATAACGCTTGTCAGCGG

AGGAACAGCAACTTTGAAGGTGGCCCCCGTAATGACCGTTCCAGTGGGGC

TCGTAACTCACTGGGTCGAAACGCACCAACTCATATTTATAGTGATCCAA

GCACTGTAAAATGCGCTAACGCTGTATTTAGTGGAATGATTGGTGGTGCG

ATCAAAGGAGGTCCCATAGGAATGGCAAGAGGTACCATTGGTGGAGCCGT

TGTTGGTCAATGTCTCTCAGATCATGGTAGTGGAAATGGAAGTGGTAACA
```

-continued

GAGGAAGTTCCAGTAGTTGTTCAGGTAATAATGTTGGCGGAACATGTAAC

CGATAA

M A N I R E L T L D E I T L V S G G N A N S N F E

G G P R N D R S S G A R N S L G R N A P T H I Y S

D P S T V K C A N A V F S G **M** I G G A I K G G P I

G **M** A R G T I G G A V V G Q C L S D H G S G N G S

G N R G S S S S C S G N N V G G T C N R Stop

cDNA sequence and protein sequence for mcpI
(SEQ ID NOS: 25 and 26)

ATGGAGGGCGCTACTATGTTTATTAAATTACTTTCCTTTATATGTGGTTT

GTTACTGGGATTTGCACTATTGAGTGGCTCCTCTGTTATTGATTTATACT

GGTTTTCACTACCTTCCGAGTTTTCAAAGATTGTAGTCATGCTGATCACT

CTTTTTTCCACGGCAAGATTCATGGACTATATCATAGAAAAAATAAGAAC

AATCTCCGCGAAATAG

M E G A T **M** F I K L L S F I C G L L L G F A L L S

G S S V I D L Y W F S L P S E F S K I V V **M** L I T

L F S T A R F **M** D Y I I E K I R T I S A K Stop

cDNA sequence and protein sequence for mcpA
(SEQ ID NOS: 27 and 28)

ATGAATGATAACATATATAAATATAGTAAAGATAATGCGATAGCGtTTCT

TCTACTTGTTGTTATATCAACAGTTGTGATATTCACACCGGCATTCACCA

TACAATATATTGGTTTGGATCTGGCATTTTCCTTTGTCTTTATTACTGAA

ATTTTAATGTCAACTTCATTTTATATTTTTTACTTAAGAAGAATACCAGG

TTGTAAAATCACCATAAAGACAAATGCGAAAACATTAAAGCTATTAGTAA

TATCATTTGCTGTGATTGCTCTCATGCAACTGCTTATTTTTGCTTATAGA

GACAATTTGAACAATAGTGAATCAACTTCACTTAATTGGATTGAAATATT

TATACTGGTCCTGACAGTTCCGTATTATGAAGAAATTGTTTACCGAACAT

GTCTATTCGGTCTTCTATGTACGACTTATaAAAAAGAATTATTTAcCCCC

TGCGTGTGTACATCTTTAtTTTTCTGCCTGATGCATCCGCAGTATTATAA

TGTGGCTGATCAAATTATTCTGTTTATTATGTCAATGTTATTGTTGAATA

TAAGGATTTGCAGTAAGGGGATTTTCTATCCAATGCTGTTACATGCGGGA

ATAAACGGCTTTGTTATATTGTTAAATATATTATAG

M N D N I Y K Y S K D N A I A F L L L V V I S T V

V I F T P A F T I Q Y I G L D L A F S F V F I T E

I L **M** S T S F Y I F Y L R R I P G C K I T I K T N

A K T L K L L V I S F A V I A L **M** Q L L I F A Y R

D N L N N S E S T S L N W I E I F I L V L T V P Y

Y E E I V Y R T C L F G L L C T T Y K K E L F T P

C V C T S L F F C L **M** H P Q Y Y N V A D Q I I L F

I **M** S **M** L L L N I R I C S K G I F Y P **M** L L H A G

I N G F V I L L N I L Stop

-continued

cDNA sequence and protein sequence for mcpD
(SEQ ID NOS: 29 and 30)

ATGAATATATTCAGAAGTGAAGCAATAGAACATCATAATGACACTGAATA

TGGTGACATTATTTTACCAACATCATTTAGCCTATCCGTATGTGCAACAG

TTACATTATTCATTATGTTAAGTCTGACTGTATTCATATATTACGGTAGC

TATACAAGGAAAGCGCATCTTACAGGTATCGTCATGCCCTCATCAGGACT

GGTAAAAATAATTCCTCAATATGCAGGATATGTAACACAACTGACTGTAT

CCGAAGGAGAACACGTAACTGCAGGGACACAACTCTATCATATAAGTGGA

GAACATTATAACGGTAACGGAACTGGCACATTAGCAACGATGAGTATTTC

CCTGAAGACTCAGTATATTATGTTGGCCTCCCAGCAATCCTTTGAGTCGC

GAGATAATAGTCAACAACAGGAAGCCATACGGCAAAGGATGATATCACTT

GAGCCGCAAATAAGAAGTGCAGAACAAAGACTTCAGCTTGCTGAACGTCA

GGCAGAACTGGCTATATCCGTCATGGAACGCTATAAAAAATTGGCTGGTA

CGCATTATGTGTCAGATATCGAATTCCAACAGAAACAAATTGATGTTTCT

GCCGCTCAACAAAACGTTGAAGATCAGCGTCAGGGGCTTCTCCAGTTACA

TACTGCAATGGACACAGCCAAAGATGAACTAAATCATCTTATTGTTCAGG

GGAAAAGCCGTAAAGCAGAACTCGACAGACAATTGCAGGTGCTAAAACAA

CAACAGGATGAACTCGCCGGACAAGAAAAATTTACACTGAGGGCTCCAGT

ATCCGGGACTATTGCTGCTGTACTGATCAAACAGGGGCAGTCTGTGAAAG

CATCTGAACCGGTCATGACTCTCATTCCCGATAATGCTCATTTACAAATT

GAGCTTTATGCTACCAGCCAGAAAGCCGGTTTTATCCGACCAGGTCAACG

GGTATCTCTGAAGTTTTCGGCCTTCCCTTATCAGAAATTTGGTATCCAGT

ACGGCACAATTCGTAAAATCAGTCATACGACTCTGGCTCCTTCCGACTTA

TTACCAGITTCACCCGTCACATGGAAAGAAAACGAAGGGCATTATCGCGT

TATTGTTGAACCTGAAAATACATTTATATTTGCATACGGaAAAAAAGAAC

CGCTAAGACCAGGCATGACTCTGGAAGGAGACGTCAACCTTGATACTCGT

CATTTATGGGAATGGCTGACAGAGCCCCTATGGAGCATGAAAGGAAATCT

GTAA

M N I F R S E A I E H H N D T E Y G D I I L P T S

F S L S V C A T V T L F I **M** L S L T V F I Y Y G S

Y T R K A H L T G I V **M** P S S G L V K I I P Q Y A

G Y V T Q L T V S E G E H V T A G T Q L Y H I S G

E H Y N G N G T G T L A T **M** S I S L K T Q Y I **M** L

A S Q Q S F E S R D N S Q Q Q E A I R Q R **M** I S L

E P Q I R S A E Q R L Q L A E R Q A E L A I S V **M**

E R Y K K L A G T H Y V S D I E F Q Q K Q I D V S

A A Q Q N V E D Q R Q G L L Q L H T A **M** D T A K D

E L N H L I V Q G K S R K A E L D R Q L Q V L K Q

Q Q D E L A G Q E K F T L R A P V S G T I A A V L

I K Q G Q S V K A S E P V **M** T L I P D N A H L Q I

E L Y A T S Q K A G F I R P G Q R V S L K F S A F

-continued

P Y Q K F G I Q Y G T I R K I S H T T L A P S D L

L P V S P V T W K E N E G H Y R V I V E P E N T F

I F A Y G K K E P L R P G **M** T L E G D V N L D T R

H L W E W L T E P L W S **M** K G N L Stop

cDNA sequence and protein sequence for mcpB
(SEQ ID NOS: 31 and 32)

ATGGAATCAATAAACTGGAAAGTAAGGAAACAACTACCCGTTATCCGTCA

AACCGAATCAGCTGAATGCGGTCTGGCGTGTCTGGCTATGATTGCCTGCT

GGCATGGACTGAAAACAGATTTATCGACATTACGGGAACGTTTCAATATA

GGTATTCAGGGAATGACGCTACAAAGGTTGATCGAATGTGCAGCGTCCAT

CCATTTATCATCACGTGCAGTTCGTCTGGAACCCGAAGATCTGAGGTGTC

TTAATCTTCCATCTATTCTGCACTGGGATATGAACCATTTCGTCGTTCTC

CATAAAGTTCGGGGAAACCGGTTATACATCCATGATCCGGACAGAGGAAA

AATTACAATAAGTCTGTTGGACGCAGGTAAGCATTTTACAGGAGTGGCAC

TGGAATTAACTCCAGCCAGTGATTTCACCCCCCGGAACGAGAGAAAAAAA

TCCACCTGCGTCAACTGACAGGGAAAACCCCGGGGCTTTTAGCATCAATG

ACaAAAATTATTATTTTTGCTCTGGCCCTTGAGATTCTGGCTTTAGGTGG

TCCACTTCTTAATCAACTGGTAATTGATGAAGTTCTGGTCGCAGCAGACA

GAAGTCTATTGTATGTCATTATAGTGGCACTACTGTTGTTATCACTCATA

CAATTATTACTCTCCCTAGCACGACAATGGGCAACGATCAGTTTATCCGT

CAATTTTAACATGCAATGGACTGCCAGAGTTTTCCATCATCTTGTAAGAC

TCCCTCTTGCATGGTTCGATGCCCGAAGTAAAGGAAGTATTAATGCCCGT

TTTGAAGCAGTAGATATAATCCAGCAGGCGCTGACAACGCAGGTTCTTGA

AGGCATTCTGGATATGCTACTTATTGTGACTGCTCTTTGCATGATGCTGT

TGTATAGCCCAGGAATGACATTAATCGCAGTAATTGCAGCTATTATATAT

GGCGCACTGAGAGCATTGTGGTATCCGGCTTTACGGCAATCTGTTGAAGA

TGTCTGGGATGCAGGAACTAAGGAGTCGGGGCATTTTCTCGAAACCCTTA

ACGGCATTCAGAGTCTGAGAATCAACGGTGTAACTATTCACAGAGAAGCG

GCCTGGCTGAACCTCAACGTTACCCGCAGAAACACACAGCTACGCCAGAA

TCGTTTACAAATGAGCTATGAACTGACGCATACACTGACGGAAAGTGTAG

TTTCAGCCATTATTTTGTGGCAGGGAGCAGTAGAAGTGCTGGATGGGACA

TTTACCGTGGGTATGTTGGTTGCTTACTTATCCTATCAGATGCGTTTTTC

ATCCAGTATAAGCAATCTGACTGATAACTTTTTTTCCTGGCGCATGCTTG

ATGTTTATAACGAGAGACTTGCCGATATTGTGCTAACACCACAGGAAGGT

CACCAGAATCAGCACCATTGGGCAAACCATAATGAAACAATATCTGCAAG

CCAGTACAGAGAACATAAATATGATAATACCCATCCACCATTACTTATCG

aAAAAATAACATTTAGCCATAAGGGCGCAGATAAACCCATATTGGATAAC

GCGTCACTAATGCTCTTTCCTGGAGAAATATTAGCAATAACAGGTAAATC

AGGATGTGGCAAATCAACATTGGTAAAGCTTATTCTTGGAATTCATACAC

CAAGTGAAGGAAGAATTAATGCATTTGGCATACCACATACACATTCTGAT

TATTTTCAGGTTCGTCAACGAATTGGCACTGTATTGCAAGATGACTATCT

TTTCAAAGGTTCTATAGCTGATAATATAATGTTTTTTAGCGAAATTAGAG

-continued

ATCATGAACACATGCGTAAATGCGCAAGTCTGGCACTTATAGACAGTGAT

ATTATGGCAATGCCAATGGGCTATCAACATTACTTGGAGAAACCGGAGGG

GGACTTTCAGGTGGTCAGAAGCAACGTATTCTACTGGCAAGAGCACTGTA

TAAAAAACCCGGTCTATTATTACTGGACGAAGCAACCAGTCATCTTGATG

TGGAAAGTGAAATAGAAATAAGCCAGACATTACGCCAACTCGGATTCCTG

TTCTGTTAATAGCTCATCGACCAGAAACAATAGCATCCGCAGACAGAGTT

CTATCTGAGAGATGGTCACTTTTCGGAAATAACATATCGACCTGCCAGAA

CTCATAATATAAATAATCACCCCAACAGGAGGTGA

M E S I N W K V R K Q L P V I R Q T E S A E C G L

A C L A **M** I A C W H G L K T D L S T L R E R F N I

G I Q G **M** T L Q R L I E C A A S I H L S S R A V R

L E P E D L R C L N L P S I L H W D **M** N H F V V L

H K V R G N R L Y I H D P D R G K I T I S L L D A

G K H F T G V A L E L T P A S D F T P R N E R K K

I H L R Q L T G K T P G L L A S **M** T K I I I F A L

A L E I L A L G G P L L N Q L V I D E V L V A A D

R S L L Y V I I V A L L L L S L I Q L L L S L A R

Q W A T I S L S V N F N **M** Q W T A R V F H H L V R

L P L A W F D A R S K G S I N A R F E A V D I I Q

Q A L T T Q V L E G I L D **M** L L I V T A L C **M** **M** L

L Y S P G **M** T L I A V I A A I I Y G A L R A L W Y

P A L R Q S V E D V W D A G T K E S G H F L E T L

N G I Q S L R I N G V T I H R E A A W L N L N V T

R R N T Q L R Q N R L Q **M** S Y E L T H T L T E S V

V S A I I L W Q G A V E V L D G T F T V G **M** L V A

Y L S Y Q **M** R F S S S I S N L T D N F F S W R **M** L

D V Y N E R L A D I V L T P Q E G H Q N Q H H W A

N H N E T I S A S Q Y R E H K Y D N T H P P L L I

E K I T F S H K G A D K P I L D N A S L **M** L F P G

E I L A I T G K S G C G K S T L V K L I L G I H T

P S E G R I N A F G I P H T H S D Y F Q V R Q R I

G T V L Q D D Y L F K G S I A D N I **M** F F S E I R

D H E H **M** R K C A S L A L I D S D I **M** A **M** P **M** G Y

Q T L L G E T G G G L S G G Q K Q R I L L A R A L

Y K K P G L L L L D E A T S H L D V E S E I E I S

Q T L R Q L G I P V L L I A H R P E T I A S A D R

V L Y L R D G H F S E I T Y R P A R T H N I N N H

P N R R

Example 10. Sequence for Plasmid Containing the Unique 4.8-Kb Operon Region that is Present in PDI$^+$ Strains but not PDI$^-$ Strains (GenBank Accession: JQ901381)

This sequence for the plasmid containing the unique 4.8-kb operon region that is present in PDI$^+$ strains but not PDI$^-$ strains was determined (GenBank accession JQ901381) and is presented as SEQ ID NO: 33. The region covering mcpM, mcpI, mcpA, mcpD, and mcpB is presented as SEQ ID NO: 34. Within SEQ ID NO: 34, the nucleotide positions of individual operon components are as follows: mcpM (41253-43442); mcpM (43443-44695); mcpA (44798-45433); mcpD (45438-45654); and mcpB (45663-46026).

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Example 11. Microcin MccPDI Reduces the Prevalence of Susceptible *Escherichia coli* in Neonatal Calves

MccPDI microcin producing *E. coli*-25 or the equivalent knockout strains were co-inoculated into calves with susceptible *E. coli*-186 to investigate the function of MccPDI in vivo. MccPDI-producing *E. coli*-25 out-competed *E. coli*-186 (P=0.003), consistent with MccPDI being responsible for antibiotic resistant *E. coli*-25 competitive advantage in calves. The increasing prevalence of antibiotic resistant bacteria presents a major challenge for both human and animal health. High levels of antimicrobial usage in livestock potentially plays an important role in amplifying and retaining antibiotic resistance genes in bacterial populations (6, 7, 13). Nevertheless, even in the absence of antibiotic use resistant bacteria can persist (2, 5, 8). A previous study showed that *E. coli* strains with resistance to streptomycin, sulfadiazine, and tetracycline (SSuT) were the dominant *E. coli* found in calves at the Washington State University (WSU) dairy (10). Mixtures of these SSuT *E. coli* isolates, including strain *E. coli*-25, demonstrated a fitness advantage in dairy calves and in broth culture over antibiotic susceptible strains (10). The mechanism allowing these strains to dominate in calves was unknown, but was not associated with antimicrobial resistance traits (9). Recently we showed *E. coli*-25 produces the novel microcin, MccPDI, that is responsible for killing susceptible *E. coli* in vitro. MccPDI-production allows *E. coli*-25 to inhibit a diversity of *E. coli*, including olates enterohemorrhagic (EHEC) and enterotoxigenic (ETEC) strains (4, 11). The spectrum of MccPDI activity makes *E. coli*-25 attractive as a probiotic against pathogenic bacteria with potential for prophylactic, therapeutic, and food safety applications. Consequently, we tested the hypothesis that an MccPDI-producing *E. coli*-25ΔtraM strain will limit colonization of dairy calves by the MccPDI-susceptible *E. coli*-186, while MccPDI-knockout strain, *E. coli*-25ΔmcpMΔmcpI would exhibit no selective advantage in vivo (4).

This study was conducted in the large animal isolation facilities at WSU under a WSU Institutional Animal Care and Use Committee approved protocol. Calves inoculated with the same strains were housed in groups when possible. Bulk milk was fed two to three times daily with one feeding containing 1 tbsp. of milk non-antibiotic containing supplement (10). Calves were pre-screened for nalidixic acid-, kanamycin-, and chloramphenicol-resistant *E. coli* using methods described below. If resistant bacteria were detected the calf was not used in the study. Kanamycin- and chloramphenicol-resistant *E. coli*-25 mutants were generated (Table 1) to allow the use of calves that carried either kanamycin or chloramphenicol resistant flora, but did not carry both. Each calf (<3 days old) was orally inoculated with 10^9 CFU of each competing *E. coli* strain. Inoculum was prepared by pelleting overnight cultures of each strain, resuspending the cells in fresh LB, and mixing the cultures immediately before inoculation. If the inoculated strains were not detected at one day post-inoculation, a second dose was administered on day two. Day one refers to the day following the final inoculation. The trial included two groups of calves with group one (n=4) receiving MccPDI knockout *E. coli*-25ΔmcpMΔmcpI and *E. coli*-186 and group two (n=7) receiving MccPDI-producing *E. coli*-25ΔtraM and *E. coli*-186; chance enrollment of calves with incompatible antibiotic resistant flora led to rejection of more calves from group one.

Fecal samples were collected directly from the rectum of each calf immediately following inoculation (day 0) and each day for six days (10). Within 4 h of collection ten-fold serial dilutions of each sample were prepared in sterile PBS and plated on MacConkey agar to determine total colony forming units (CFUs) of lactose fermenting enteric bacteria with colony morphology consistent with *E. coli*. MacConkey agar supplemented with kanamycin (50 μg/ml) and tetracycline (50 μg/ml), chloramphenicol (34 μg/ml) and tetracycline (50 μg/ml), or nalidixic acid (30 μg/ml) was used to enumerate the test strains present in the fecal samples. A competition index (CI) was calculated to compare the fitness of competing strains (FIG. **5**). PCR was used to confirm the identity of the *E. coli*-25 mutants by pairing primers within the resistance cassette (3) with locus-specific primers (Table 3; n=368). The putative *E. coli*-186 isolates (n=172) recovered from the fecal samples were confirmed by pulsed-field gel electrophoresis analysis (1).

TABLE 3

E. coli strains and PCR primer sequences used in this work.

E. coli Strain	Genotype/ phenotype	Strain-specific primer	Ref.
25ΔmcpM ΔmcpI	SSuT^r Chlor^r ΔmcpMΔmcpI PDI$^-$	mcpM_mcpI fwd: CAAACAACCGATAGGGGAAA (SEQ IN NO: 36) c2: GATCTTCCGTCACAGGTAGG (SEQ IN NO: 37)	This work
25ΔmcpM ΔmcpI	SSuT^r Kan^r ΔmcpMΔmcpI PDI$^-$	mcpM_mcpI fwd: CAAACAACCGATAGGGGAAA (SEQ IN NO: 38) k2: CGGTGCCCTGAATGAATGAACTGC (SEQ IN NO: 39)	(4)
25ΔtraM	SSuT^r Chlor^r ΔtraM PDI$^+$	traM fwd: GTTCTGCCATCCTGCGTTAT (SEQ IN NO: 40) c1: TTATACGCAAGGCGACAAGG (SEQ IN NO: 41)	This work
25ΔtraM	SSuT^r Kan^r ΔtraM PDI$^+$	traM fwd: GTTCTGCCATCCTGCGTTAT (SEQ IN NO: 42) k1: CAGTCATAGCCGAATAGCCT (SEQ IN NO: 43)	(4)
186	Wild-type; Nal^r PDI$^-$		(11)
O157: H7 6-E12057	Wild-type; Nal^r Cip^r	rfb fwd: AAGATTGCGCTGAAGCCTTT (SEQ IN NO: 36) rfb rvs: CATTGGCATCGTGTGGACAG (SEQ IN NO: 36)	(12)

At six days post-inoculation calves were euthanized and five to ten centimeter lengths of the cecum, spiral colon, descending colon, and rectal-anal junction (RAJ) were collected. All fecal matter was removed by rinsing the tissue in sterile PBS. A 6 mm sterile biopsy punch was used to collect a sample and make a 1:10 dilution (sa/vol) in PBS. The tissue was homogenized, serially diluted, and plated onto MacConkey agar supplemented with antibiotics.

A previous study with *E. coli*-25 indicated the microcin-producing strain *E. coli*-25ΔtraM should have a distinct advantage over the susceptible strain *E. coli*-186 (10). As expected, by day six, *E. coli*-25ΔtraM dominated *E. coli*-186 (CI=0.22, P=0.003; FIG. **1**). In contrast, the microcin-knockout strain *E. coli*-25ΔmcpMΔmcpI was significantly less fit than *E. coli*-186 (CI=−0.95; FIG. **5**). Notably, different treatment outcomes were asymmetric with the CI for *E. coli*-25ΔtraM (0.22) being a smaller magnitude than the competition index for *E. coli*-25ΔmcpMΔmcpI (−0.95). This could possibly be explained by the presence of native MccPDI-expressing strains that would also compete with *E. coli*-25ΔtraM while enhancing inhibition of the susceptible *E. coli*-25ΔmcpMΔmcpI.

If most native *E. coli* strains are susceptible to MccPDI, and if the MccPDI producing strain has a fitness advantage relative to non-producing strains of *E. coli*, then the MccPDI-producing strain should be found in greater numbers relative to the total *E. coli* population. We enumerated the CFU for the *E. coli*-25 mutants relative to the CFU lactose-fermenting enteric bacteria in the fecal samples. *E. coli*-25ΔmcpMΔmcpI accounted for <0.2% of the total lactose-fermenting enteric bacteria by day six, whereas *E. coli*-25ΔtraM consistently comprised >2% of this population throughout the trial (repeated measures ANOVA, P=0.01; FIG. **6**). There was no difference between the total number of lactose-fermenting bacteria between the two groups (P=0.96). These results confirm the MccPDI-producing strain has a selective advantage over the non-producing strain in this model.

E. coli was consistently recovered from tissues of the lower gastrointestinal tract (GI). The inoculated strains, however, were typically only detected at counts just above the detection level. *E. coli*-25ΔtraM strain was recovered more frequently compared to *E. coli*-186, whereas *E. coli*-25ΔmcpMΔmcpI was recovered at a frequency similar to the *E. coli*-186 strain (P=0.01 and P=0.052, respectively; paired t-test; FIGS. **7**A and B). There were no apparent differences in the frequency of detection between tissues (ANOVA, P=0.41). These results are consistent with *E. coli*-25ΔtraM having an in vivo fitness advantage allowing better colonization within calves.

Sixteen arbitrarily selected *E. coli* from each calf were tested for strain identity. Of calves inoculated with *E. coli*-25ΔtraM and *E. coli*-186, we detected the expected strains in 5 and 6 calves, respectively. We only recovered the expected strains from 1 of 4 calves for the non-microcin treatment group.

Bacteriocin-producing bacteria present an attractive means to control pathogens in food animal production. *E. coli*-25ΔtraM reduces the shedding of *E. coli*-186 confirming that MccPDI is functional in vivo. Changing the timing and doses of *E. coli*-25ΔtraM may potentially improve the treatment effect because it is unknown when or at what concentration MccPDI functions in vivo. Future research should investigate the use of multiple bacteriocinogenic strains, increased doses, or pre-inoculation of *E. coli*-25ΔtraM to limit pathogenic *E. coli* populations in cattle.

References for Example 11

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Example 12. Identification of a Receptor and Associated Proteins Required for MccPDI to Recognize and Inhibit Susceptible *E. coli*

The *E. coli* Keio Collection, a single-gene deletion library, was screened for mutants able to grow in the presence of the MccPDI producing strain *E. coli*-25. The Keio Collection includes individual gene knockouts for all non-essential genes that are expressed by *E. coli* strain BW25113, which is also sensitive to PDI. Screening of the full library followed by verification experiments demonstrated that mutants of atpA, atpF, dsbA, dsbB, ompF, or ompR were no longer sensitive to PDI, indicating these genes are required for MccPDI function.

Materials and Methods

Strains and Culture Conditions.

E. coli-25 [streptomycinR, sulfadiazineR, tetracyclineR, (SSuTR)] (26), *E. coli* S17, *E. coli* BW25113 and the *E. coli* BW25113 gene-deletion library (Keio Collection, kanamycinR, Thermo Scientific) were used in this study. *E. coli* BW25113 was purchased from the *Coli* Genetic Stock Center (CGSC, Yale) and it is susceptible to antibiotics employed in the current study. To isolate this strain in a mixed culture, *E. coli* BW25113 was made nalidixic acid resistant by passaging 5 times with increasing concentrations until it was capable of growing in 30 μg/ml nalidixic acid. Unless stated otherwise, all strains were cultured in either Luria Broth (LB) or M9 Minimal Media at 37° C. shaking 250 rpm. Antibiotics were used at the following concentrations: tetracycline (50 μg/ml), chloramphenicol (34 μg/ml), kanamycin (50 ng/ml), nalidixic acid (30 μg/ml) and ampicillin (100 μg/ml).

Screening the Keio Collection for Loss of PDI.

The Keio collection of *E. coli* knockouts (Thermo Scientific) was employed to identify genes associated with susceptibility to MccPDI. Each mutant was grown overnight at 37° C. without shaking in a 96-well plate containing 150 μl fresh LB (50 ng/ml kanamycin) per well. A 10 ml culture of *E. coli*-25 was also started at this time in LB (50 μg/ml tetracycline) and incubated at 37 C with shaking (250 rpm). The following day each mutant was individually placed into co-culture with *E. coli*-25 in a sterile, U-bottom 96-well plate with 200 μl M9 minimal media per well. A 96-pin replicator (Boekel Scientific) was used to transfer overnight cultures (~1 μl) of each strain for competition experiments. The replicator was sterilized **3**X between each use by submerging pins into 70% ethanol and flaming. The *E. coli*-25 culture was poured into a sterile plastic trough and transferred in the same manner. Competition cultures were incubated overnight at 37° C., shaking at 100 rpm. Approximately 24 h later co-cultures (~1 μl) were transferred onto LB agar containing kanamycin or tetracycline to select for the Keio strains or *E. coli*-25, respectively. The plates were incubated at 37° C. for at least 6 h. Growth on tetracycline verified the presence of *E. coli*-25 in the culture. No growth on kanamycin indicated that the Keio knockout strain being tested was still susceptible to PDI. Growth of a Keio mutant strain on the kanamycin plate indicated putative identification of gene knockouts that were no longer susceptible to PDI.

Competition Assays and Enumeration of Resistant *E. coli* Mutants.

To confirm detection of PDI-resistant strains from the Keio collection, prospective strains were grown overnight in LB with antibiotic selection. Each mutant strain culture was then added (10 μl) with *E. coli*-25 culture (10 μl) to 2 ml M9 media. These co-cultures were incubated at 37° C. for 8 to 24 h. To determine the CFUs of each strain following competition, serial dilutions of the co-cultures were prepared in a 96-well plate containing sterile PBS and then spotted (5 μl) onto LB agar supplemented with kanamycin or tetracycline. Agar plates (3 per enumerated dilution) were incubated overnight at 37° C. and colonies were tallied for total colony forming units (cfu).

PCR Verification of the Knockout Mutants.

Primers corresponding to sequence up- and down-stream the deleted gene (Table 3) were designed to verify the location of the kanamycin cassette insertion. Reactions were carried out with an initial denaturing step at 95° C. for 4 min, followed by 30 cycles of 95° C. for 20 s, 55° C. for 20 s, 72° C. for 1 min 20 s, and a final extension at 72° C. for 5 min. The PCR products were evaluated using electrophoresis alongside an O'gene Ruler 1 Kb plus ladder (Thermo Scientific) to determine the amplicon sizes. The wild-type (non-mutant) *E. coli* BW25113 was included as a control in addition to a no-template negative control.

Regenerating Resistant Mutants in *E. coli* S17.

To validate the findings from the Keio library screen, we generated independent mutants in a PDI sensitive strain, *E. coli* S17. The methods used to create these mutants were previously described by Datsenko and Wanner (2000; Proc Natl Acad Sci USA 97:6640-5) and were the same used to create the Keio Collection (Baba et al. Mol Syst Biol 2:2006 0008). The new gene-deletion mutants were generated with the insertion of chloramphenicol resistance (cat) instead of a kanamycin cassette as was used for the Keio Collection. PCR primers were designed to amplify the chloramphenicol cassette from plasmid pKD3 and contained extensions identical to the sequence flanking the gene of interest (Table 3). *E. coli* S17 was transformed with the pKD46 plasmid, which facilitates homologous recombination between the gene of interest and the PCR amplicon. *E. coli* S17+pKD46 was grown in super optimal broth (2% bacto-trypton, 0.5% yeast extract, 8.56 mM NaCl, 2.5 mM KCL, 10 mM $MgCl_2$) with ampicillin and 0.1 mM arabinose to induce the proteins necessary for homologous recombination. These induced cells were then made competent and electroporated with the chloramphenicol amplicon containing the requisite flanking sequences. Transformed cells were recovered at 30° C. and were plated onto LB agar with chloramphenicol to select for successful deletion mutants. PCR was used to verify the insertion site of the chloramphenicol cassette (Table 3) using PCR conditions as describe above.

Growth Curves.

All strains, including the gene knockout mutants, were assessed for their ability to grow in M9 and LB media. Growth curves were run on a BioScreen C (Oy Growth Curves Ab Ltd). Each strain was grown individually overnight in LB media with antibiotic selection. These cultures were used to inoculate (1:1,000 dilution) M9 minimal media or LB broth without antibiotics. Cultures incubated for 24 h at 37° C. with continuous shaking and optical density (450-580 nm) measured every 30 min.

Fluorescent Labeling and Microscopy.

E. coli-25ΔmcpMΔmcpI, *E. coli*-25ΔtraM, and *E. coli*-186 were each transformed with a vector expressing cherry red fluorescent protein (pFPV-mCherry) or GFP (pFPV25). Competition assays (described above) were conducted with the fluorescently labeled cells; one with MccPDI-producing *E. coli*-25ΔtraM+pFPV-mCherry and PDI-susceptible *E. coli*-186+pFPV25, and another with MccPDI—nonproducing *E. coli*-25ΔmcpMΔmcpI+pFPV-mCherry and PDI-susceptible *E. coli*-186+pFPV25. Additionally, these competitions were repeated where each strain carried the opposite plasmid to ensure the results were not caused by either strain differentially expressing either fluorescent protein. Individual cultures were run as controls. Each culture was visualized at 24 h using a fluorescent scope at 60× magnification.

Results

Six *E. coli* mutants in the Keio Collection were resistant to PDI. The single gene deletion *E. coli* mutant library, called the Keio Collection, was used to identify genes putatively associated with susceptibility to MccPDI. Approximately 3,985 mutants were screened using a high-throughput 96-well plate method. Following two rounds of screening using these methods, six mutants were identified as potentially being resistant to inhibition by *E. coli*-25. These mutants were then placed into a 2 ml competition experiment (M9 media) with *E. coli*-25 and CFUs were subsequently enumerated after 24 h co-culture. Mutants that were able to grow to a population density >10^5 CFU were considered resistant to PDI and these included the mutants with a deletion in atpA, atpF, dsbA, dsbB, ompF, or ompR (Table 4).

TABLE 4

Gene-knockout mutants that are no longer susceptible to killing by *E. coli*-25. PCR primer sequences amplify the gene of interest and were used to verify the specific knockouts. The CFUs represent the average of triplicate competition assays.

Gene Disrupted	Primers: Gene-specific primers and flanking sequences (H1[a] and H2[b])	Gene Function	Co-culture with *E. coli*-25 (CFU)
atpA	atpA fwd: TGCTGCGATGGAAAAACGTC (SEQ ID NO: 36) atpA rvs: TTCTGGACGCTTGCGATCTT (SEQ ID NO: 37) H1: CTTGCAGACGTCTTGCAGTCTTAAGGGGACTGGAGC (SEQ ID NO: 38) H2: GCCTGCGGCCTGCCCTAAGGCAAGCCGCCAGACGT (SEQ ID NO: 39)	ATP synthase, F1 complex, α subunit; also called papA	4.10×10^5
atpF	atpF fwd: ATCGCTGTAGGTCTGGGTCT (SEQ ID NO: 40) atpF rvs: ATGTCCTGCCAGCGTTCTAC (SEQ ID NO: 41) H1: AATATCAGAACGTTAACTAAATAGAGGCATTGTGCT (SEQ ID NO: 42) H2: CTACCGTAATAAATTCAGACATCAGCCCCTCCCTCC (SEQ ID NO: 43)	ATP synthase, F0 complex, b subunit; also called papF	6.51×10^6

TABLE 4-continued

Gene-knockout mutants that are no longer susceptible to killing by *E. coli*-25. PCR primer sequences amplify the gene of interest and were used to verify the specific knockouts. The CFUs represent the average of triplicate competition assays.

Gene Disrupted	Primers: Gene-specific primers and flanking sequences (H1[a] and H2[b])	Gene Function	Co-culture with *E. coli*-25 (CFU)
dsbA	dsbA fwd: AGCGGCAGGATGCATTATCA (SEQ ID NO: 44) dsbA rvs: GGGAAGATTACTGGCTGCGA (SEQ ID NO: 45) H1: GTGAATATTCACGGGCTTTATGTAATTTACATTGAA (SEQ ID NO: 46) H2: AATTAACACCIATGTATTAATCGGAGAGAGTAGATC (SEQ ID NO: 47)	Periplasmic protein disulfide isomerase (disulfide bond formation)	2.37×10^7
dsbB	dsbB fwd: CAATGGCAGATGAAGCGAGC (SEQ ID NO: 48) dsbB rvs: TGCAAATGGGCTGGATAGCA (SEQ ID NO: 49) H1: AACTGCGCACTCTATGCATATTGCAGGGAAATGATT (SEQ ID NO: 50) H2: CAGGAAAAAAGCGCTCCCGCAGGAGCGCTGAAGGGA (SEQ ID NO: 51)	Disulfide oxidoreductase (disulfide bond formation) membrane protein; oxidizes periplasmic DsbA	3.97×10^7
ompF	ompF fwd: CGCTATCAGGGTAACGGGAG (SEQ ID NO: 52) ompF rvs: AGCACTTTCACGGTAGCGAA (SEQ ID NO: 53) H1: GTTGTCAGAATCGATCTGGTTGATGATGTAGTCAAC (SEQ ID NO: 54) H2: GTGATCGTCCCTGCTCTGTTAGTAGCAGGTACTGCA (SEQ ID NO: 55)	Outer membrane protein, porin	9.50×10^6
ompR	ompR fwd: TGTTGCGAACCTTTGGGAGT (SEQ ID NO: 56) ompR rvs: AGCAAGGTGACGATGAGCAA (SEQ ID NO: 57) H1: GGGCAAATGAACTTCGTGGCGAGAAGCGCAATCGCC (SEQ ID NO: 58) H2: CTTACAAATTGTTGCGAACCTTTGGGAGTACAAACA (SEQ ID NO: 59)	Transcriptional regulatory protein OmpR; Response regulator for osmoregulation	2.12×10^6

[a] *E. coli* S17 gene-specific sequences are shown. For gene deletion mutants, flanking sequences also included the chloramphenicol primer site: TGTGTAGGCTGGAGCTGCTTCG, (SEQ ID NO: 60) 3' to the *E. coli* S17 specific sequence.

[b] *E. coli*-25 gene-specific sequences are shown. For gene deletion mutants, flanking sequences also included the chloramphenicol primer site: CATATGAATATCCTCCTTA, (SEQ ID NO: 61) 3' to the *E. coli* S17 specific sequence.

Example 13. Microcin PDI Regulation and Post-Translational Modifications are Unique Among Known Microcins

In this study, we used competition assays with gene knockout and complemented strains, QPCR assays, western blots and electrophoretic mobility assays to demonstrate that expression of the microcin PDI is induced in low osmolarity conditions and is regulated by the EnvZ/OmpR system through the binding of the phosphorylated OmpR to the mcpM promoter region. The phosphorylated OmpR may recognize three different binding sites within this promoter region. This system of regulation is similar to that of OmpF and unique from other described microcins. Site-directed mutagenesis revealed that the McpM precursor peptide includes two leader peptides that undergo sequential cleavage (positions G17/G18 and G35/A36) during export through the type I secretion system. Competition assays showed that these cleaved products are required for the PDI phenotype. McpM has four cysteines within the mature peptide and experimental data showed that the first two cysteines are necessary for the microcin PDI inhibition of susceptible cells. DAPI staining of fluorescently-labeled strains showed that the microcin PDI likely induces membrane permeability of susceptible cells. Without being bound by theory, the data suggests that McpM forms a multimer within the periplasm of susceptible cells where it damages membrane integrity. Together these data combined with previous work indicates that MccPDI is unique amongst the microcins that have been described to date.

Introduction

Like bacteriocins from Gram-positive bacteria, microcins are generally derived from precursor peptides that are composed of a C-terminal core region and an N-terminal leader peptide. The leader peptide is typically cleaved during the process of export (Kolter & Moreno, 1992, Duquesne et al., 2007). For example, the Colicin V precursor protein (a class IIa microcin) includes a double-glycine leader peptide, consisting of 15 amino acid residues, which is cleaved during export by the CvaA/CvaB/TolC export machinery (Gilson et al., 1990) (Havarstein et al., 1994; Zhong et al., 1996).

Microcin PDI (MccPDI), a newly identified microcin, is of particular interest because it inhibits the growth of a broad diversity of *E. coli* including EHEC serotypes O157:H7 and O26 (Sawant et al., 2011). The inhibitory phenotype has been called "proximity-dependent inhibition" (PDI) because inhibition only occurs when the microcin-producing cells are located in close proximity to sensitive cells (Sawant et al., 2011). Whole-genome sequence analysis has identified five open-reading frames: mcpM and mcpA (microcin synthesis), mcpI (immunity) and mcpD and mcpB (export) (Eberhart et al., 2012). The number and organization of the genes resembles that of the class IIa microcins. Gene deletions verified that five plasmid encoded genes and the chromosomally located tolC are responsible for the PDI phenotype. To date, however, gene regulation, protein maturation and protein function have not been determined for MccPDI.

In this study, we demonstrate that the expression of microcin MccPDI is responsive to extracellular osmolarity and is regulated by the EnvZ/OmpR system. Maturation of the MccPDI effector protein, McpM, involves two sequential cleavage events, and once exported it appears that McpM interacts with susceptible cells resulting in increased cell membrane permeability.

Material and Methods

Bacterial Strains, Media and Growth Conditions.

E. coli strains were cultured in LB-Lennox medium (LB broth) (Difco) or in M9 minimal medium (6 g/L Na2HPO4, 3 g/L KH2PO4, 0.5 g/L NaCl, 1 g/L NH4Cl, 2 mg/L thiamine, 1 mM MgSO4, 0.1 mM CaCl2, and 0.2% glucose) at 37° C. with shaking (200 rpm). Unless otherwise indicated, antibiotics were added to media at the following concentrations: tetracycline (Tet), 50 μg/ml; chloramphenicol (Cm), 34 μg/ml; kanamycin (Kan), 50 μg/ml; nalidixic acid (Nal), 30 μg/ml; and ampicillin (Amp), 100 μg/ml. LB broth with different salt concentrations were made by mixing 10 g/L Bacto-tryptone, 5 g/L yeast extract and NaCl at indicated concentrations.

DNA Manipulation and Mutant Construction.

Extraction of *E. coli* genomic DNA was accomplished using a Dneasy® Blood & Tissue kit following the manufacturer's instruction (Qiagen). Plasmid DNA was purified using a QIAprep® Spin Miniprep Kit (Qiagen). Conventional PCR included DreamTaq® Green PCR Master Mix (Thermo Scientific) while preparative PCR was used for plasmid construction using Platinum PCR SuperMix High Fidelity (Invitrogen) according to the manufacturer's protocol. Deletion cassettes for chromosomal in-frame deletions were first generated using the splice-overlap-extension method (Heckman & Pease, 2007), which joins two 400-600 bp PCR fragments corresponding to genomic sequences flanking the gene(s) of interest. The deletion cassettes were subsequently cloned into a suicide plasmid (pDM4) by using standard cloning procedures (Milton et al., 1996) followed by confirmation with DNA sequencing. The resulting constructs were individually electroporated into *E. coli* S17-1 λpir, after which they were introduced by conjugation into MccPDI-producing *E. coli*-25. Mutant strains were selected on LB plates containing Cm and Tet followed by a 10% sucrose selection process. Gene deletion was confirmed by PCR using primers located just outside of the deleted sequence. Plasmids for complementation (pMMB207 and pCR2.1) and overexpression (pPAL7) were constructed by using standard cloning procedures and all inserts were fully sequenced to confirm construct assembly. For site-directed mutagenesis, primers were designed by using (EbaseChanger and were then used to generate point mutation plasmids with a Q5 Site-Directed Mutagenesis Kit (New England Biolabs) following the manufacturer's protocol. These constructs were introduced into their target strains by electroporation.

Competition Assays.

Bacterial strains were grown individually overnight in LB media with appropriate antibiotics. Equal volumes of each competing strain were inoculated at 1:200 into either fresh LB medium with different salt concentrations or M9 medium. The cultures were mixed and incubated at 37° C. for 12 h. When necessary, IPTG (100 μM unless specified otherwise) and antibiotics (chloramphenicol or ampicillin) were added during the competition. Monocultures of each competing strain were also prepared as controls by inoculation into the appropriate media at the same dilution. To estimate the number of colony forming units (CFUs) for each strain following competition, a 6×6 drop-plate method was employed (Chen et al., 2003) with triplicate counts for each competition experiment (technical replicates were averaged before analysis).

RNA Isolation and qPCR.

Expression was quantified for mcpA, mcpB, mcpM, mcpI and tolC at 4, 8, 12 and 24 h. Briefly, a cell pellet was collected by centrifugation from 1.0-1.5 Ml broth culture. This was resuspended in RNAwiz® reagent (350 μL; Bacteria Ribopure kit; Ambion). Primary organic extraction was carried out as per manufacturer's instructions. The RNA was treated with RQ1-RNase-free DNase (Promega) for 30 min at 37° C., followed by a second organic extraction using TRIzol LS (Invitrogen) as per manufacturer's instructions. The final RNA was quantified using a NanoDrop™ 2000 Spectrophotometer (Thermo Scientific). All RNA extractions were confirmed as "DNA free" by subjecting them to a qPCR reaction with primers for rpoD (without cDNA synthesis). Any samples for which a Ct value of <38 cycles was generated were treated a second time with DNase and were re-extracted as described above. RT reactions were performed utilizing iScript Supermix (BioRad) as per manufacturer's instructions with 500 ng of RNA in a total volume of 20 μL. The resultant cDNA was diluted 1:10 with the addition of 180 μL of ultra-pure water. Diluted cDNA (5 μL) was used as template in each qPCR reaction. qPCR reactions included SsoAdvance SYBR Mastermix (2×) (BioRad). Mastermix (10 μL) was combined with 5 μL of cDNA template and 200 nM of each primer in a final volume of 20 μL. All primer pairs were run using the same cycling parameters: initial denaturation at 95° C. for 2 min, followed by 40 cycles of 55° C. for 1 min and 95° C. for 15 s. Fluorescent signal was recorded during the annealing/extension step (55° C.). A melt-curve analysis was performed on all reactions starting at 75° C. and increasing 0.5° C./cycle, with a pause and fluorescence detection at each temperature for 5 s. All assays were run in triplicate and each condition was run in biological duplicate. rpoD served as the housekeeping gene for normalization purposes.

Electrophoretic Mobility Shift Assays (EMSA).

DNA fragments 1-8 (see results) were prepared by PCR and were then purified by using a QIAquick® PCR purification kit (Qiagen). Fragments 9-10 were obtained by annealing oligonucleotides in annealing buffer (10 mM Tris pH 8.0, 50 mM NaCl, 1 mM EDTA). Briefly, equal volumes of complementary oligonucleotides (at equimolar concentration) were mixed in a 1.5 ml microfuge tube and placed in a heat block at 95° C. for 5 min. The heat block along, with the samples, was removed from the apparatus and allowed to cool for 1 h to room temperature. The resulting double-stranded DNA was separated on a 2.0% agarose gel and purified using QIAquick gel extraction kit (Qiagen). All fragments were quantified using a NanoDrop™ 2000 Spectrophotometer before EMSA experiments. The OmpR and XRE (control) proteins were expressed and purified using the Profinity eXact System. Concentration of purified proteins was estimated using a micro-BCA protein assay kit (Thermo Scientific). The purified proteins were mixed with the DNA fragments at different concentrations in 20 μL of binding buffer [10 mM Tris (pH 7.5), 100 mM KCl, 10 mM MgCl2, 1 mM DTT, 5% glycerol]. Binding reactions were incubated at room temperature for 30 min before adding 5 μL of 5× loading buffer. The samples were electrophoresed on 5% native TBE gels (BioRad) for 45 min at 100 V followed by staining with ethidium bromide.

Western Blot Analysis.

Protein samples from bacterial pellets and cell fractions were denatured in boiling water for 5 min in tricine sample buffer (BioRad). SDS-PAGE was used to separate proteins with either Any kD Tris-glycine precast gels or a 16.5% Tris-Tricine precast gels (BioRad) prior to western blotting. The Tris-Tricine gels were used to improve resolution for McpM. A Trans-Blot turbo transfer starter system (BioRad) was used to transfer proteins to a low-fluorescence polyvinylidene fluoride (LF-PVDF) membrane. Primary antibodies anti-Histag (1:2500, Novagen), anti-DnaK (1:5000, Abcam) were used with secondary goat anti-mouse antibody (1:5000, DyLight 488 conjugate) to visualize proteins on western blots. A ChemiDoc MP Imaging System (BioRad) was used to detect fluorescent signal.

Supernatant Protein Precipitation.

Bacterial strains were grown 10 h at 37° C. (200 rpm) in M9 broth (50 ml) supplemented with appropriate antibiotics. Supernatants were filtered through 0.45-μm PVDF syringe filters, and the proteins in the supernatant were precipitated by adding 20% (vol/vol) trichloroacetic acid (TCA) followed by incubation on ice for 1 h. Precipitated protein was pelleted by centrifugation (12,000×g for 1 h), washed with acetone for 15 min, dried, and suspended in Tricine sample buffer.

Fluorescent Labeling and DAPI Staining.

To construct pFPV-tdTomato, the gfpmut3 gene in pFPV25.1 (Valdivia & Falkow, 1996) was replaced with the tdTomato gene (Clontech) using primers tdtomato_XbaI and tdtomato_SphI. Standard cloning procedures were used and sequencing was used to verify results. E25ΔtraM:kan and E25ΔmcpMΔmcpI:kan were transformed with the pFPV25.1-gfpmut3 expressing green fluorescent protein (GFP), while target strain BW25113 was transformed with the pFPV-tdtomato expressing red fluorescent protein (tomato red). Competition assays (described above) were conducted with the fluorescently labeled cells, and individual monocultures were run as controls. After 2 h and 6 h incubations, 1 ml samples were taken from each of the cultures and 4,6-Diamidino-2-phenylindole dihydrochloride (DAPI) (Thermo Scientific) stain was added at a final concentration of 0.25 μg/ml for 10 min at room temperature. The stained bacteria were pelleted by centrifugation for 1 min at 12,000×g and re-suspended into the same volume of PBS buffer. Cells were then immobilized onto poly-L-lysine coated glass slides (Sigma) for 20 min and covered with glass coverslips. Cells were observed and images were captured by using an inverted fluorescence microscope (EVOS, Advanced Microscopy Group).

Statistical Analysis.

All qPCR results were processed using the A-A Ct method (Livak & Schmittgen, 2001) with the resultant fold change/biological replicate analyzed using an ANOVA with a Bonferroni multiple comparison post-hoc test (NCSS 2007; LLC. Kaysville, Utah). Other comparisons were made by using ANOVA with a Dunnett's one-way multiple comparisons posthoc test (SigmaPlot version 12.5; Systat Software, Inc., San Jose, Calif.).

Results

MccPDI is Regulated by the EnvZ/OmpR Two-Component Regulatory System.

A previous study reported that inhibition by MccPDI was phenotypically obvious when strains were co-cultured in M9 medium (0.05% NaCl) but muted when co-cultured in LB medium (0.5% NaCl) (Sawant et al., 2011). Competition assays were performed here between a ccPDI producing strain (*E. coli*-25) and a target strain (BW25113) in M9 and LB with different concentrations of NaCl. We found that the MccPDI phenotype was significantly enhanced in LB containing low (0.05% NaCl) or no added salt (FIG. **9**A). In contrast, when the concentration of NaCl in LB or M9 was increased (0.5% and 1%), inhibition was reduced correspondingly, indicating that MccPDI function or synthesis is responsive to changes in osmolarity. Consistent with the phenotype, qPCR analysis revealed that transcription of mcpM was significantly upregulated at 8 h in M9 when compared to LB (FIG. **10**B). Transcription of other genes (mcpI, mcpA, and mcpB) within MccPDI gene cluster showed a similar pattern when cultured in LB and M9 media.

The *E. coli* EnvZ/OmpR two-component system responds to osmolarity changes in broth media (Forst & Roberts, 1994, Cai & Inouye, 2002). To determine if this system is involved with MccPDI regulation, envZ and ompR deletion strains were constructed in *E. coli*-25. Transcription of mcpM was down-regulated significantly, particularly at 4 h, for the E25ΔenvZ strain when compared to the isogenic control (FIG. **10**A). Competition assays showed that the envZ or ompR gene knockouts abolished inhibition from MccPDI whereas complementation of each gene in the corresponding mutant restored the PDI phenotype (FIG. **9**B). Furthermore, when the functionally important histidine residue (H243) of EnvZ was mutated to alanine, complementation using this mutant envZ sequence did not restore the inhibition phenotype (FIG. **10**B). This data is consistent with MccPDI being regulated by osmolarity that is signaled through the EnvZ/OmpR two-component system.

Phosphorylated OmpR Binds to the Promoter Region of mcpM.

EnvZ is an osmotic sensor that regulates the phosphorylation state of the transcriptional factor OmpR (Qin et al., 2000). Phosphorylated OmpR controls the expression levels of outer membrane porin genes (ompF and ompC) and other virulence and fimbriae genes by binding their promoter region (Cai & Inouye, 2002, Feng et al., 2003, Jubelin et al., 2005). To determine if the OmpR binds to the promoter sequence of mcpM, a 200 bp DNA fragment located at position −10 bp to −210 bp (Pmic-10/-210) relative to the start codon of mcpM was mixed with recombinant OmpR and subjected to a mobility-shift assay. A concentration-dependent shift of the OmpR/DNA mixture was clearly evident and is consistent with OmpR binding to the mcpM promoter region. The negative control protein, XRE (expressed and purified identically to recombinant OmpR) did not bind the promoter of mcpM. A secondary control involving an unrelated DNA fragment (270 bp, atpE) displayed no gel shift after addition of purified OmpR. Sequence analysis showed that there is a non-coding region upstream from the ABC transporter genes (mcpD and mcpB) that could serve as another promoter sequence within the MccPDI gene cluster. No gel shift was observed for this 143 bp DNA fragment (PmicD-20/-163; relative to mcpD start codon), implying that the fragment PmicB-20/-163 does not contain a promoter cassette that can be recognized by OmpR.

Additionally, no gel shift was observed for another 201 bp DNA sequence (Pmic-233/-433; relative to mcpM start codon).

To determine if OmpR phosphorylation is required for binding to the mcpM promoter region, we mutated a conserved D55 residue of OmpR that is known to undergo transphosphorylation by EnvZ (Forst et al., 1994). This mutated OmpR recombinant protein was mixed with Pmic-10/-210 and the gel mobility shift assay showed no evidence of binding even when 900 ng of protein was added. Consistent with the data, the OmpR, purified from M9 media, which contained a reduced amount of phosphorylated OmpR, displayed reduced binding to the mcpM promoter region compared to the OmpR purified from LB media. Taken together, this data indicates that phosphorylated OmpR binds to the promoter region of mcpM.

mcpM promoter region includes more than one OmpR recognition site. To identify the sequence motif(s) that OmpR binds to within Pmic-10/-210, a series of truncated DNA fragments were prepared (FIG. **11**A). OmpR bound DNA fragments 1-3, but not fragments 4 and 5, indicating that the region between −81 to −102 is necessary for OmpR binding. This is consistent for the gel shifts that were evident for fragments 6-8 and fragment 10, which contain the region between −81 to −102, whereas fragment 9 did not bind as expected. From a qualitative perspective, binding was reduced for fragments 3, 7 and 8 compared with fragments 1, 2, and 6 (the same batch of recombinant OmpR was used for all of these mixtures). This could be a staining artefact (less ethidium bromide intercalated into shorter strands of DNA), but this is also consistent with sequences −61 to −81 and −102 to −134 providing additional binding sites or stabilizing OmpR binding. The mcpM promoter region is rich in adenines and thymines and three possible binding sites (B1, B2 and B3) are highlighted based on the above data (FIG. **11**B). A multiple sequence alignment showed that the three possible binding sites, especially B1, resemble the consensus OmpR binding sites for ompF and ompC (F1, F2, F3, F4 and C1) and include conserved nucleotides that are important for OmpR binding (Harlocker et al., 1995). Collectively, these data are consistent with the mcpM promoter region having multiple binding sites and that region B1 is likely the primary binding site for the OmpR protein. McpM is cleaved into three peptides. Eberhart et al. (2012) could not recover the MccPDI inhibition phenotype with complementation of ΔmcpM:Kan. They assumed this was due to a polar effect on the immunity protein, mcpI, owing to the proximal insertion of a kanamycin resistance gene. For this reason, we generated a new scarless knock-out of mcpM using the suicide vector pDM4 in the wild-type *E. coli*-25 strain. As expected, the inhibition phenotype was lost for the new knockout strain, but we were able to restore the phenotype upon in trans expression of mcpM driven by an IPTG-induced promoter (Ptac) in pMMB207 vector (FIG. **12**). Importantly, western blot analysis showed three distinct protein products in the mcpM complemented strains. The upper band is the presumptive fulllength protein because its mass is consistent with the mass of the recombinant protein (also visible when synthesized in two MccPDI-susceptible strains, *E. coli* BW25113 and 186). Presumably, the middle and lower bands are cleaved forms of the full length protein. To exclude the possibility that the promoter (Ptac) affects the observed phenotype, we generated a recombinant mcpM construct with the native promoter (Pmic-1/-210+mcp/14). The new construct displayed the same phenotype as the previous construct (p207::mcpM) that was driven by Ptac promoter (FIG. **12**).

McpM Undergoes Two Cleavage Events.

Class II microcins are typically generated from a precursor protein that harbors conserved leader peptides (Duquesne et al., 2007). Alignment of class IIa microcin precursors showed that a conserved double glycine is present in positions 17 and 18 of mcpM, consistent with the presence of an 18-residue leader peptide (FIG. **13**). When the glycine residues were changed to proline (G17P and G18P) this resulted in loss of two protein bands relative to the wild-type strain. Changing only one glycine to alanine (G17A or G18A) did not prevent cleavage although the faint-low mass protein bands are consistent with reduced cleavage efficiency for the G17A mutation. Double mutations from glycine to alanine (G17A/G18A) abolished cleavage. Competition assays showed that these mutants are unable to inhibit sensitive strains (FIG. **14**).

If changes to the double-glycine site (G17/G18) results in loss of a cleavage site, this would only explain one of the two proteins band differences. One possibility is that the smallest protein band is the cleaved product while the middle band is modified form of the cleaved product. Peptide sequencing after different protease treatments (trypsin, chymotrypsin, and elastase) and analysis using UPLC-MS/MS on an Q-Exactive Orbitrap instrument showed no evidence for post-translational modifications (Bioproximity), arguing that the middle band is not a modified form of the smaller protein (data not shown). Alternatively, a second double glycine (positions 26 and 27) or a glycine-alanine motif at positions 35 and 36 could be the second cleavage sites for McpM (FIG. **13**). Mutations G26P and G27P had no effect on the cleavage while mutations G35P and A36D resulted in loss of the lower-mass protein band.

These data indicate that MccPDI has two leader peptides (1-18 and 19-36) and the protein undergoes two cleavage events during maturation which are likely sequential. A competition assay employing the mutated secondary cleavage site (G35P or A36D) displayed only partial inhibitory activity when compared with wild-type strain (FIG. **14**). We also mutated other residues (R5, E11, V15, S16, N19, S33, R37 and G41) located within the two leader peptides and around the cleavage sites. Western blots showed that each mutant was processed normally, although E11A and V15A may have resulted in reduced synthesis of MccPDI. All mutants, except N19 exhibited comparable inhibition of a susceptible strain (FIG. **15**).

Cleavage of McpM is Insufficient to Produce a Functional Protein.

We generated different constructs of mcpM that lack individual leader peptides (Δ1-18 or Δ19-36) or both (Δ1-36; FIG. **16**A). Neither the Δ1-18 or Δ1-36 strains inhibited sensitive strains (FIG. **16**B). Furthermore, western blots showed very limited quantities of McpM from these strains compared to the wild-type strain. The Colicin V leader peptide shares ~50% amino acid identity with the primary leader sequence of McpM (FIG. **13**). Consequently, we therefore replaced the primary leader peptide with the Colicin V leader peptide (ColV1-15/Δ1-18) (FIG. **16**A), but this was not sufficient to recover wild-type phenotype (FIG. **16**B) and very limited quantities of McpM were again detected by western blot.

Interestingly, the Δ19-36 strain appeared to have a similar concentration of protein as the wild-type strain, but its inhibitory activity was severely reduced (FIG. **16**B). It is possible that the reduced activity is due to loss of export owing to the missing second leader sequence. Nevertheless, a western blot demonstrated that the product was present in

TCA-precipitated culture supernatant. Both of the cleaved McpM products appeared to be present for the wild-type strain whereas no products were present for the secretion-negative ΔmcpB strain. While the A19-36 construct of McpM is exported, it appears to have lost most of its functional activity (FIG. **16**B, last bar).

McpM Cleavage is Concomitant with Export.

Class II microcin export machinery displays a canonical structure consisting of three components. The ABC transporter and an accessory protein are encoded in the microcin gene cluster while the third component is the chromosome-encoded TolC (Vassiliadis et al., 2011). We verified that ΔmcpB and ΔmcpD strains lose the PDI phenotype and complementation restores it (FIG. **12**). Sequence alignment shows that McpB contains three conserved domains including an N-terminal peptidase C39 domain, an ABC transporter transmembrane domain, and a C-terminal ABC transporter ATP-binding domain, which is consistent with the ABC transporter family. In trans expression of mcpM in a double knockout (ΔmcpMΔmcpB) demonstrated that deletion of mcpB leads to the loss of McpM cleavage, confirming that the ABC transporter is responsible for cleavage of this protein. McpD is homologous to proteins of class II microcin export machinery and it likely serves as a connector between the ABC transporter and TolC (Gilson et al., 1990, Pons et al., 2004). In trans expression of mcpM in the double knockout ΔmcpMΔmcpD did not affect production of full-length McpM, but cleavage was blocked as with the ΔmcpB strain, indicating that cleavage of McpM is concomitant with export.

The First Two Cysteines within the McpM are Necessary for Function.

In class IIa microcins, cysteine pairs are commonly associated with the formation of disulfide bonds (Duquesne et al., 2007). The McpM protein includes four cysteine residues (positions 57, 90, 109 and 118) and all are located within the mature peptide sequence (FIG. **13**). To determine if these cysteine residues are involved in post-translational modifications, each was individually mutated to alanine using site-directed mutagenesis. Western blots demonstrated that these point mutations did not affect McpM synthesis or post-translational cleavage, but the PDI inhibitory phenotype was eliminated for C57A and C90A, whereas mutation of cysteines 3 and 4 (C109A and C118A) had no affect on function (FIG. **17**). These results are consistent with the possibility that a disulfide bond is required between the cysteine residues located at positions 57 and 90 for McpM to be functional. DsbA and DsbB are thiol-redox enzymes that are responsible for disulfide-bond formation in *E. coli* (Inaba, 2009). Knockouts of dsbA and dsbB (ΔdsbA and ΔdsbB) in the wild-type E-25 strain did not result in loss of PDI phenotype (FIG. **18**). Furthermore, a series of double-knockouts (ΔdsbAΔdsbB and ΔdsbAΔdsbD) or triple-knockout (ΔdsbAΔdsbBΔdsbD) eliminated the possible redundancy between the DsbA/DsbB and DsbC/DsbD pathways, but did not impact the killing phenotype strain (FIG. **18**).

MccPDI Induces Membrane Permeability.

To probe how MccPDI exerts its inhibitory activity on target cells, we fluorescently labeled the mccPDI-producing strain (GFP-labeled E25ΔtraM and E25ΔmcpMΔmcp I) and target strain (tdTomato-labeled *E. coli* BW25113) to distinguish the two populations. After co-culture for 2 and 6 h, the cell mixtures were stained with a low concentration of DAPI (0.25 μg/ml). DAPI is normally used to stain fixed cells that have a permeabalized cell membrane. Although high concentration of DAPI can also be used to stain live cells, the effectiveness of the stain is lower (Zink et al., 2003). MccPDI-susceptible cells were co-cultured with E25ΔtraM for 6 hours after which they were easily stained by using a low concentration of DAPI (FIG. **19**, top right panel). As a control, the susceptible cells were co-cultured with E25ΔmcpMΔmcpI for 6 hours and after applying the same staining protocol, there was no DAPI staining observed (FIG. **19**, bottom right panel). DAPI also did not stain either strain in monoculture (data not shown).

Discussion

Regulation of Microcin PDI.

Bacteriocin production is an inducible process that is affected by different environmental and nutritional factors (Drider et al., 2006). For example, expression of colicin genes is regulated by the SOS response regulon that responds to DNA damage (Walker, 1995, Gillor et al., 2008). Alternatively, regulation of microcin synthesis is more related to nutrient depletion or anoxic conditions (Duquesne et al., 2007). For example, production of many class I microcins (MccB17, MccC7/C51 and MccJ25) are upregulated when cells reach the stationary growth phase (Moreno et al., 2002). One notable exception is MccE492, which is only produced during the exponential growth phase (Corsini et al., 2002). Nitrogen starvation induces MccB17 production (Connell et al., 1987) and MccV production is initiated under iron-limiting conditions (Gilson et al., 1990).

MccPDI gene expression increases rapidly during log-phase growth and drops off as the population enters stationary phase (Eberhart et al., 2012). The PDI phenotype is enhanced significantly when these experiments are conducted in M9 media compared to LB media, arguing that differences between the media (e.g., salt concentrations) might affect microcin synthesis or function (Sawant et al., 2011). We demonstrated that osmolarity in the growth media regulates expression of the mcpM. This is a novel regulatory mechanism with respect to what is known about microcins, although osmolarity can influence bacteriocin production in Gram-positive bacteria (Uguen et al., 1999).

The EnvZ/OmpR two-component regulatory system plays a central role in mediating the response to osmotic stress in *E. coli* (Stock et al., 2000). Consequently, it was not surprising to find that osmolarity-sensitive expression of MccPDI is dependent on the EnvZ/OmpR system where the phosphorylated transcriptional regulator, OmpR, binds to the mcpM promoter region. Similarly, Hernandez-Chico et al (1986) reported that expression of MccB17 gene cluster was dependent on the OmpR transcriptional factor, but this regulation is growth-phase dependent. RNA polymerase sigma 70 factor (δ70) is involved in MccB17 expression (Bohannon et al., 1991) while RNA polymerase sigma S (δS) appears to regulate MccJ25 and mccC7/C51. The histonelike protein H-NS acted as a repressor of MccB17 and mccC7/C51 expression (Fomenko et al. 2001; Moreno et al., 2002).

The EnvZ/OmpR system also regulates synthesis of the outer membrane proteins OmpF and OmpC that enable bacteria to cope with fluctuations in osmolarity (Forst et al., 1989). Under high osmolarity conditions, EnvZ auto-phosphorylates and transfers the phosphoryl group to OmpR, producing the phosphorylated form OmpR-P. At low osmolarity OmpR-P is present in low concentrations. OmpR-P binds to the promoter regions of outer membrane porin genes ompF and ompC and differentially modulates their expression according to the concentration of cellular OmpR-P (Yoshida et al., 2002). There are several binding sites for OmpR-P within the promoter region of ompF. When present in low concentrations, OmpR-P only binds to the high-affinity sites. Under high osmolarity conditions, OmpR-P concentration increases and binding occurs at low-affinity sites that result in reduced expression of ompF (Harlocker et al., 1995). Here, we show that regulation of mcpM is negatively correlated with osmolarity of the growth media (greater in M9 than LB). OmpR-P clearly binds the promoter region of mcpM whereas unphosphorylated OmpR does not. Consequently, the EnvZ/OmpR system is required for activation of MccPDI and we propose that mcpM transcriptional regulation mirrors that of ompF regulation. This conclusion is further supported by the finding of at least three putative binding sites in the MccPDI promoter region that resembles the consensus OmpR binding site for ompF.

Furthermore, Zhao et al. (2015) recently demonstrated that McpM interacts with OmpF and consequently, the concurrent expression of these traits in producer and susceptible cells likely maximizes the ability of the MccPDI-producing strains to inhibit susceptible competitors. McpM maturation. Functional microcins are usually derived from a precursor protein that is composed of a C-terminal structural region and an N-terminal leader peptide (Kolter Moreno, 1992). Enzymatic cleavage removes the leader peptide and the microcin may or may not undergo further post-translational modification. The Class II microcins have conserved leader peptides that range in size from 15 to 19 residues and harbor a double-glycine or glycinealanine cleavage site (Duquesne et al., 2007). In contrast, there is little sequence similarity between the leader peptides of Class I microcins. For example, the MccB17 precursor, a class I microcins, is processed at G26, but this cleavage site is not a typical sequence of the doubleglycine-type leader peptides as described for class II microcins (Davagnino et al., 1986).

MccPDI most closely resembles a Class IIa microcin based on its genetic organization (Eberhart et al., 2012). The complete microcin protein, McpM, contains a typical double-glycine cleavage site (G17G18) and a conserved leader peptide (residues 1-18) similar to other Class II microcins. McpM also harbors a second cleavage site (G35A36) corresponding to a second leader peptide sequence. To our knowledge, this is the first report of a microcin containing two leader peptides (1-18 and 19-36).

Leader peptides typically prevent microcin function (e.g., in the cytoplasm of the producing strain) or serve as a recognition site for export (Drider et al., 2006). For McpM, experimental evidence suggests that the first leader peptide, but not the second, is required for export. The first leader sequence may also serve to inhibit protein degradation because when absent we find only very small quantities of the modified McpM protein in the cell. We assume that the second cleavage event takes place during or after export. If the latter, this would be consistent with the hypothesis that the fully functional microcin is composed of a dimer or multimer of the two cleaved products.

Cysteine is the least abundant amino acid found in proteins (Brooks & Fresco, 2002) and it performs a variety of essential functions including binding metal ions and forming disulfide bonds that produce three-dimensional protein structures (Giles et al., 2003). For these reasons, if a protein contains an "even" number of cysteines and is predicted to function outside the cytoplasm, it is likely that cysteines form disulfide bonds (Berkmen, 2012). McpM has four cysteines within the mature protein, consistent with the prediction that disulfide bond formation occurs. Our experimental data showed that the first two cysteines are necessary for MccPDI inhibition. We also conducted competition assays in the presence of 5 mM DTT, a reducing agent that breaks disulfide bonds. Under these conditions, no inhibition was observed (data not shown) although this type of experiment could have multiple confounding effects. There was no evidence in this study that Dsb-based enzymatic activity in the McpM-producing strain contributes to disulfide-bond formation, but other works shows that strains lacking DsbA or DsbB are less susceptible to MccPDI (Zhao et al., 2015). While a computational three dimensional model for McpM did not support the formation of an intra-molecular disulfide bond between cysteine 57 and cysteine 90 due to the physical distance between these sites (data not shown), the reduced killing activity observed when only one of the two cleaved forms is present suggests the possibility of inter-molecular disulfide-bond formation. If disulfide bonds are required for function, we surmise that they form after the mature McpM protein enters the susceptible cell where folding likely occurs in the periplasm.

For class IIa microcins, cysteines commonly form disulfide bonds in the mature peptide. The full-length MccB17 protein has four cysteine residues that form heterocyclic rings by an unusual post-translational modification of the mature microcin (Bayer et al., 1995), and mutational analysis suggests that the mature form of MccV has a disulfide bond between the cysteine residues at positions 76 and 87 (Zhang et al., 1995). In addition, using mass spectrometry Pons et al. (2004) detected the presence of two intramolecular disulfide bridges in the mature MccL.

After maturation and export, microcins inhibit susceptible bacteria through a variety of mechanisms. MccJ25 recognizes the outer membrane protein FhuA and requires the inner membrane proteins TonB, ExbB, ExbD and SbmA, for translocation (Destoumieux-Garzon et al., 2005, Salomon & Farias, 1993, Salomon & Farias, 1995). Once it reaches the cytoplasm. MccJ25 inhibits transcription by obstructing the RNA polymerase secondary channel (Wilson et al., 2003). MccB17 binds OmpF on the outer membrane and the inner protein SbmA mediates uptake into the cytoplasm, where MccB17 inhibits the DNA gyrase (Lavina et al., 1986).

Microcin C7/C51 requires OmpF and the inner-membrane ABC-transporter, Yej, to be actively transported through the inner membrane (Novikova et al., 2007). Within the target cell MccC7/C51 is cleaved to form a modified aspartyl adenylate that inhibits Asp-tRNA synthetase, thus blocking protein synthesis at the translation level (Kazakov et al., 2008, Metlitskaya et al., 2006). MccE492, MccM, and MccH47, all Class IIb microcins, are unable to inhibit the growth of strains carrying mutations in the fepA, cir, and fiu genes, consistent with the requirement for these iron-catecholate receptors (Thomas et al., 2004, Patzer et al., 2003).

The transport of class IIb microcins across the outer membrane is also TonB-dependent (Destoumieux-Garzon et al., 2006, Thomas et al., 2004). Once in the periplasm, MccE492 functions by inserting into the inner membrane and interfering with membrane potential (Lagos et al., 1993, Destoumieux-Garzon et al., 2003). This activity is facilitated by the inner membrane proteins ManY and ManZ (Bieler et al., 2006). MccH47 exerts its activity by inhibiting the ATP synthase (Trujillo et al., 2001). MccV causes channel formation and disruption of membrane potential by binding to the inner membrane receptor SdaC (Yang & Konisky, 1984, Gerard et al., 2005). Recently, it was shown that MccPDI interacts with the sole receptor OmpF (Zhao et al., 2015). In this study we further demonstrate that MccPDI undergoes two sequential cleavage events, with the mature microcin inducing membrane permeability in susceptible cells. Without being bound by theory, the following model is representative of MccPDI function. First, McpM precursor protein undergoes two cleavage events to produce two cleaved forms during and possibly after export. The two cleaved peptides interact with OmpF of susceptible cells, cross the outer membrane using an unknown mechanism to access the periplasm where disulfide bridges facilitate the formation of multimers. The disulfide bonds are formed utilizing the target cells thio-redox systems, DsbA/B and/or DsbC/D, and once fully mature, the multimers permeablize the susceptible-cells membrane leading to cell death.

References for Example 13

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Example 14. McpM Functions as an Independent Protein

Based on our prior findings, it is clear that McpM is the "effector" protein for mccPDI. This soluble protein is excreted via a type I secretion system where it interacts with the OmpF protein that is displayed on the surface of susceptible *E. coli*. We generated plasmid constructs that encode mcpM ("M" for full-length McpM and two additional versions "82" and "109" that lack signal peptides) with a histidine "tag" allowing purification of the recombinant protein using conventional chromatography techniques. The recombinant proteins were incubated at room temperature with 0%, 10% or 20% DMSO to enhance the efficiency of potential spontaneous disulfide-bond formation within and between the recombinant protein molecules. These preparations were then "spotted" onto bacterial "lawns" composed of a mccPDI-sensitive strain of *E. coli*. In FIG. **20**A, faint zones of clearance are visible regardless of pre-incubation with 0%, 10% and 20% DMSO (see arrows; DMSO-only controls are at bottom of plate) and regardless of the construct that was used. FIG. **20**B shows a similar experiment with six replicate spots of an independent batch of full-length, recombinant McpM that was pre-incubated in 20% DMSO. The variance in zone clearance between the FIGS. **20**A and B is attributable to differences in recombinant protein concentration or in the agar media (M9 FIG. **20**A, LB FIG. **20**B). Regardless, these results demonstrate that the recombinant McpM inhibits growth of susceptible *E. coli* independent of a McpM-producing *E. coli* strain.

```
                              SEQUENCE LISTING


<160> NUMBER OF SEQ ID NOS: 77

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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 1

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<210> SEQ ID NO 2
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 2

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<210> SEQ ID NO 3
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 3

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<210> SEQ ID NO 4
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

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<210> SEQ ID NO 5
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 5
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ccgtaatgac cgttccagt 19

<210> SEQ ID NO 6
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 6

ccatttccac taccatgatc t 21

<210> SEQ ID NO 7
<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 7

ataacccgta tctttacgtt gccttacgtt ca 32

<210> SEQ ID NO 8
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 8

ctagaatccg caataatttt acagtttgat 30

<210> SEQ ID NO 9
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 9

aataacgtga ttgcatatta cttatctcag gagttc 36

<210> SEQ ID NO 10
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 10

atccctggaa ggactacaac ctatgaccga aaatac 36

<210> SEQ ID NO 11
<211> LENGTH: 40
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 11

gtaatttaat aaacatagta gcgccctcca ttatatctat 40

<210> SEQ ID NO 12
<211> LENGTH: 40
<212> TYPE: DNA

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 12

aacgcacaaa ataacaaaca accgataggg gaaatatgat                            40


<210> SEQ ID NO 13
<211> LENGTH: 40
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 13

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<210> SEQ ID NO 14
<211> LENGTH: 40
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 14

aacgcacaaa ataacaaaca accgataggg gaaatatgat                            40


<210> SEQ ID NO 15
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 15

tggtgatgaa ttcctgtcaa a                                                21


<210> SEQ ID NO 16
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 16

taccagtttc acccgtcaca                                                  20


<210> SEQ ID NO 17
<211> LENGTH: 38
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 17

tcagccattc ccataaatga cgagtatcaa ggttgacg                              38


<210> SEQ ID NO 18
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 18

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```

```
<210> SEQ ID NO 19
<211> LENGTH: 40
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 19

gatatacatc tgacctgtgt gatgttaaag ttttatacta                           40


<210> SEQ ID NO 20
<211> LENGTH: 40
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 20

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<210> SEQ ID NO 21
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer site

<400> SEQUENCE: 21

tgtgtaggct ggagctgctt cg                                              22


<210> SEQ ID NO 22
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer site

<400> SEQUENCE: 22

catatgaata tcctcctta                                                  19


<210> SEQ ID NO 23
<211> LENGTH: 356
<212> TYPE: DNA
<213> ORGANISM: Escherichia coli

<400> SEQUENCE: 23

atggcaaata taagagaatt aactttagat gagataacgc ttgtcagcgg aggaacagca     60

actttgaagg tggcccccgt aatgaccgtt ccagtggggc tcgtaactca ctgggtcgaa    120

acgcaccaac tcatatttat agtgatccaa gcactgtaaa atgcgctaac gctgtattta    180

gtggaatgat tggtggtgcg atcaaaggag gtcccatagg aatggcaaga ggtaccattg    240

gtggagccgt tgttggtcaa tgtctctcag atcatggtag tggaaatgga agtggtaaca    300

gaggaagttc cagtagttgt tcaggtaata atgttggcgg aacatgtaac cgataa        356


<210> SEQ ID NO 24
<211> LENGTH: 120
<212> TYPE: PRT
<213> ORGANISM: Escherichia coli

<400> SEQUENCE: 24

Met Ala Asn Ile Arg Glu Leu Thr Leu Asp Glu Ile Thr Leu Val Ser
1               5                   10                  15
```

```
Gly Gly Asn Ala Asn Ser Asn Phe Glu Gly Gly Pro Arg Asn Asp Arg
            20                  25                  30

Ser Ser Gly Ala Arg Asn Ser Leu Gly Arg Asn Ala Pro Thr His Ile
        35                  40                  45

Tyr Ser Asp Pro Ser Thr Val Lys Cys Ala Asn Ala Val Phe Ser Gly
    50                  55                  60

Met Ile Gly Gly Ala Ile Lys Gly Gly Pro Ile Gly Met Ala Arg Gly
65                  70                  75                  80

Thr Ile Gly Gly Ala Val Val Gly Gln Cys Leu Ser Asp His Gly Ser
                85                  90                  95

Gly Asn Gly Ser Gly Asn Arg Gly Ser Ser Ser Ser Cys Ser Gly Asn
            100                 105                 110

Asn Val Gly Gly Thr Cys Asn Arg
        115                 120
```

```
<210> SEQ ID NO 25
<211> LENGTH: 216
<212> TYPE: DNA
<213> ORGANISM: Escherichia coli

<400> SEQUENCE: 25

atggagggcg ctactatgtt tattaaatta ctttccttta tatgtggttt gttactggga      60

tttgcactat tgagtggctc ctctgttatt gatttatact ggttttcact accttccgag     120

ttttcaaaga ttgtagtcat gctgatcact ctttttttcca cggcaagatt catggactat     180

atcatagaaa aaataagaac aatctccgcg aaatag                                216
```

```
<210> SEQ ID NO 26
<211> LENGTH: 71
<212> TYPE: PRT
<213> ORGANISM: Escherichia coli

<400> SEQUENCE: 26

Met Glu Gly Ala Thr Met Phe Ile Lys Leu Leu Ser Phe Ile Cys Gly
1               5                   10                  15

Leu Leu Leu Gly Phe Ala Leu Leu Ser Gly Ser Ser Val Ile Asp Leu
            20                  25                  30

Tyr Trp Phe Ser Leu Pro Ser Glu Phe Ser Lys Ile Val Val Met Leu
        35                  40                  45

Ile Thr Leu Phe Ser Thr Ala Arg Phe Met Asp Tyr Ile Ile Glu Lys
    50                  55                  60

Ile Arg Thr Ile Ser Ala Lys
65                  70
```

```
<210> SEQ ID NO 27
<211> LENGTH: 636
<212> TYPE: DNA
<213> ORGANISM: Escherichia coli

<400> SEQUENCE: 27

atgaatgata acatatataa atatagtaaa gataatgcga tagcgtttct tctacttgtt      60

gttatatcaa cagttgtgat attcacaccg gcattcacca tacaatatat tggtttggat     120

ctggcatttt cctttgtctt tattactgaa attttaatgt caacttcatt ttatattttt     180

tacttaagaa gaataccagg ttgtaaaatc accataaaga caaatgcgaa aacattaaag     240

ctattagtaa tatcatttgc tgtgattgct ctcatgcaac tgcttatttt tgcttataga     300
```

```
gacaatttga acaatagtga atcaacttca cttaattgga ttgaaatatt tatactggtc    360

ctgacagttc cgtattatga agaaattgtt taccgaacat gtctattcgg tcttctatgt    420

acgacttata aaaaagaatt atttaccccc tgcgtgtgta catctttatt tttctgcctg    480

atgcatccgc agtattataa tgtggctgat caaattattc tgtttattat gtcaatgtta    540

ttgttgaata taaggatttg cagtaagggg attttctatc caatgctgtt acatgcggga    600

ataaacggct ttgttatatt gttaaatata ttatag                              636


<210> SEQ ID NO 28
<211> LENGTH: 211
<212> TYPE: PRT
<213> ORGANISM: Escherichia coli

<400> SEQUENCE: 28

Met Asn Asp Asn Ile Tyr Lys Tyr Ser Lys Asp Asn Ala Ile Ala Phe
1               5                   10                  15

Leu Leu Leu Val Val Ile Ser Thr Val Val Ile Phe Thr Pro Ala Phe
            20                  25                  30

Thr Ile Gln Tyr Ile Gly Leu Asp Leu Ala Phe Ser Phe Val Phe Ile
        35                  40                  45

Thr Glu Ile Leu Met Ser Thr Ser Phe Tyr Ile Phe Tyr Leu Arg Arg
    50                  55                  60

Ile Pro Gly Cys Lys Ile Thr Ile Lys Thr Asn Ala Lys Thr Leu Lys
65                  70                  75                  80

Leu Leu Val Ile Ser Phe Ala Val Ile Ala Leu Met Gln Leu Leu Ile
                85                  90                  95

Phe Ala Tyr Arg Asp Asn Leu Asn Asn Ser Glu Ser Thr Ser Leu Asn
            100                 105                 110

Trp Ile Glu Ile Phe Ile Leu Val Leu Thr Val Pro Tyr Tyr Glu Glu
        115                 120                 125

Ile Val Tyr Arg Thr Cys Leu Phe Gly Leu Leu Cys Thr Thr Tyr Lys
    130                 135                 140

Lys Glu Leu Phe Thr Pro Cys Val Cys Thr Ser Leu Phe Phe Cys Leu
145                 150                 155                 160

Met His Pro Gln Tyr Tyr Asn Val Ala Asp Gln Ile Ile Leu Phe Ile
                165                 170                 175

Met Ser Met Leu Leu Leu Asn Ile Arg Ile Cys Ser Lys Gly Ile Phe
            180                 185                 190

Tyr Pro Met Leu Leu His Ala Gly Ile Asn Gly Phe Val Ile Leu Leu
        195                 200                 205

Asn Ile Leu
    210


<210> SEQ ID NO 29
<211> LENGTH: 1254
<212> TYPE: DNA
<213> ORGANISM: Escherichia coli

<400> SEQUENCE: 29

atgaatatat tcagaagtga agcaatagaa catcataatg acactgaata tggtgacatt     60

attttaccaa catcatttag cctatccgta tgtgcaacag ttacattatt cattatgtta    120

agtctgactg tattcatata ttacggtagc tatacaagga aagcgcatct tacaggtatc    180

gtcatgccct catcaggact ggtaaaaata attcctcaat atgcaggata tgtaacacaa    240

ctgactgtat ccgaaggaga acacgtaact gcagggacac aactctatca tataagtgga    300
```

```
gaacattata acggtaacgg aactggcaca ttagcaacga tgagtatttc cctgaagact    360

cagtatatta tgttggcctc ccagcaatcc tttgagtcgc gagataatag tcaacaacag    420

gaagccatac ggcaaaggat gatatcactt gagccgcaaa taagaagtgc agaacaaaga    480

cttcagcttg ctgaacgtca ggcagaactg gctatatccg tcatggaacg ctataaaaaa    540

ttggctggta cgcattatgt gtcagatatc gaattccaac agaaacaaat tgatgtttct    600

gccgctcaac aaaacgttga agatcagcgt caggggcttc tccagttaca tactgcaatg    660

gacacagcca aagatgaact aaatcatctt attgttcagg ggaaaagccg taaagcagaa    720

ctcgacagac aattgcaggt gctaaaacaa caacaggatg aactcgccgg acaagaaaaa    780

tttacactga gggctccagt atccgggact attgctgctg tactgatcaa acaggggcag    840

tctgtgaaag catctgaacc ggtcatgact ctcattcccg ataatgctca tttacaaatt    900

gagctttatg ctaccagcca gaaagccggt tttatccgac caggtcaacg ggtatctctg    960

aagttttcgg ccttccctta tcagaaattt ggtatccagt acggcacaat tcgtaaaatc   1020

agtcatacga ctctggctcc ttccgactta ttaccagttt cacccgtcac atggaaagaa   1080

aacgaagggc attatcgcgt tattgttgaa cctgaaaata catttatatt tgcatacgga   1140

aaaaaagaac cgctaagacc aggcatgact ctggaaggag acgtcaacct tgatactcgt   1200

catttatggg aatggctgac agagccccta tggagcatga aaggaaatct gtaa         1254


<210> SEQ ID NO 30
<211> LENGTH: 417
<212> TYPE: PRT
<213> ORGANISM: Escherichia coli

<400> SEQUENCE: 30

Met Asn Ile Phe Arg Ser Glu Ala Ile Glu His His Asn Asp Thr Glu
1               5                   10                  15

Tyr Gly Asp Ile Ile Leu Pro Thr Ser Phe Ser Leu Ser Val Cys Ala
            20                  25                  30

Thr Val Thr Leu Phe Ile Met Leu Ser Leu Thr Val Phe Ile Tyr Tyr
        35                  40                  45

Gly Ser Tyr Thr Arg Lys Ala His Leu Thr Gly Ile Val Met Pro Ser
    50                  55                  60

Ser Gly Leu Val Lys Ile Ile Pro Gln Tyr Ala Gly Tyr Val Thr Gln
65                  70                  75                  80

Leu Thr Val Ser Glu Gly Glu His Val Thr Ala Gly Thr Gln Leu Tyr
                85                  90                  95

His Ile Ser Gly Glu His Tyr Asn Gly Asn Gly Thr Gly Thr Leu Ala
            100                 105                 110

Thr Met Ser Ile Ser Leu Lys Thr Gln Tyr Ile Met Leu Ala Ser Gln
        115                 120                 125

Gln Ser Phe Glu Ser Arg Asp Asn Ser Gln Gln Gln Glu Ala Ile Arg
    130                 135                 140

Gln Arg Met Ile Ser Leu Glu Pro Gln Ile Arg Ser Ala Glu Gln Arg
145                 150                 155                 160

Leu Gln Leu Ala Glu Arg Gln Ala Glu Leu Ala Ile Ser Val Met Glu
                165                 170                 175

Arg Tyr Lys Lys Leu Ala Gly Thr His Tyr Val Ser Asp Ile Glu Phe
            180                 185                 190

Gln Gln Lys Gln Ile Asp Val Ser Ala Ala Gln Gln Asn Val Glu Asp
        195                 200                 205
```

```
Gln Arg Gln Gly Leu Leu Gln Leu His Thr Ala Met Asp Thr Ala Lys
    210                 215                 220

Asp Glu Leu Asn His Leu Ile Val Gln Gly Lys Ser Arg Lys Ala Glu
225                 230                 235                 240

Leu Asp Arg Gln Leu Gln Val Leu Lys Gln Gln Gln Asp Glu Leu Ala
                245                 250                 255

Gly Gln Glu Lys Phe Thr Leu Arg Ala Pro Val Ser Gly Thr Ile Ala
            260                 265                 270

Ala Val Leu Ile Lys Gln Gly Gln Ser Val Lys Ala Ser Glu Pro Val
        275                 280                 285

Met Thr Leu Ile Pro Asp Asn Ala His Leu Gln Ile Glu Leu Tyr Ala
    290                 295                 300

Thr Ser Gln Lys Ala Gly Phe Ile Arg Pro Gly Gln Arg Val Ser Leu
305                 310                 315                 320

Lys Phe Ser Ala Phe Pro Tyr Gln Lys Phe Gly Ile Gln Tyr Gly Thr
                325                 330                 335

Ile Arg Lys Ile Ser His Thr Thr Leu Ala Pro Ser Asp Leu Leu Pro
            340                 345                 350

Val Ser Pro Val Thr Trp Lys Glu Asn Glu Gly His Tyr Arg Val Ile
        355                 360                 365

Val Glu Pro Glu Asn Thr Phe Ile Phe Ala Tyr Gly Lys Lys Glu Pro
    370                 375                 380

Leu Arg Pro Gly Met Thr Leu Glu Gly Asp Val Asn Leu Asp Thr Arg
385                 390                 395                 400

His Leu Trp Glu Trp Leu Thr Glu Pro Leu Trp Ser Met Lys Gly Asn
                405                 410                 415

Leu
```

<210> SEQ ID NO 31
<211> LENGTH: 2185
<212> TYPE: DNA
<213> ORGANISM: Escherichia coli

<400> SEQUENCE: 31

```
atggaatcaa taaactggaa agtaaggaaa caactacccg ttatccgtca aaccgaatca      60

gctgaatgcg gtctggcgtg tctggctatg attgcctgct ggcatggact gaaaacagat     120

ttatcgacat tacgggaacg tttcaatata ggtattcagg gaatgacgct acaaaggttg     180

atcgaatgtg cagcgtccat ccatttatca tcacgtgcag ttcgtctgga acccgaagat     240

ctgaggtgtc ttaatcttcc atctattctg cactgggata tgaaccattt cgtcgttctc     300

cataaagttc ggggaaaccg gttatacatc catgatccgg acagaggaaa aattacaata     360

agtctgttgg acgcaggtaa gcattttaca ggagtggcac tggaattaac tccagccagt     420

gatttcaccc cccggaacga gagaaaaaaa tccacctgcg tcaactgaca gggaaaaccc     480

cggggctttt agcatcaatg acaaaaatta ttatttttgc tctggccctt gagattctgg     540

ctttaggtgg tccacttctt aatcaactgg taattgatga agttctggtc gcagcagaca     600

gaagtctatt gtatgtcatt atagtggcac tactgttgtt atcactcata caattattac     660

tctccctagc acgacaatgg gcaacgatca gtttatccgt caattttaac atgcaatgga     720

ctgccagagt tttccatcat cttgtaagac tccctcttgc atggttcgat gcccgaagta     780

aaggaagtat taatgcccgt tttgaagcag tagatataat ccagcaggcg ctgacaacgc     840

aggttcttga aggcattctg gatatgctac ttattgtgac tgctctttgc atgatgctgt     900
```

```
tgtatagccc aggaatgaca ttaatcgcag taattgcagc tattatatat ggcgcactga    960

gagcattgtg gtatccggct ttacggcaat ctgttgaaga tgtctgggat gcaggaacta   1020

aggagtcggg gcattttctc gaaaccctta acggcattca gagtctgaga atcaacggtg   1080

taactattca cagagaagcg gcctggctga acctcaacgt tacccgcaga aacacacagc   1140

tacgccagaa tcgtttacaa atgagctatg aactgacgca tacactgacg gaaagtgtag   1200

tttcagccat tattttgtgg cagggagcag tagaagtgct ggatgggaca tttaccgtgg   1260

gtatgttggt tgcttactta tcctatcaga tgcgtttttc atccagtata agcaatctga   1320

ctgataactt tttttcctgg cgcatgcttg atgtttataa cgagagactt gccgatattg   1380

tgctaacacc acaggaaggt caccagaatc agcaccattg ggcaaaccat aatgaaacaa   1440

tatctgcaag ccagtacaga gaacataaat atgataatac ccatccacca ttacttatcg   1500

aaaaaataac atttagccat aagggcgcag ataaacccat attggataac gcgtcactaa   1560

tgctctttcc tggagaaata ttagcaataa caggtaaatc aggatgtggc aaatcaacat   1620

tggtaaagct tattcttgga attcatacac caagtgaagg aagaattaat gcatttggca   1680

taccacatac acattctgat tattttcagg ttcgtcaacg aattggcact gtattgcaag   1740

atgactatct tttcaaaggt tctatagctg ataatataat gtttttagc gaaattagag   1800

atcatgaaca catgcgtaaa tgcgcaagtc tggcacttat agacagtgat attatggcaa   1860

tgccaatggg ctatcaacat tacttggaga aaccggaggg ggactttcag gtggtcagaa   1920

gcaacgtatt ctactggcaa gagcactgta taaaaaaccc ggtctattat tactggacga   1980

agcaaccagt catcttgatg tggaaagtga aatagaaata agccagacat tacgccaact   2040

cggattcctg ttctgttaat agctcatcga ccagaaacaa tagcatccgc agacagagtt   2100

ctatctgaga gatggtcact tttcggaaat aacatatcga cctgccagaa ctcataatat   2160

aaataatcac cccaacagga ggtga                                         2185
```

<210> SEQ ID NO 32
<211> LENGTH: 729
<212> TYPE: PRT
<213> ORGANISM: Escherichia coli

<400> SEQUENCE: 32

```
Met Glu Ser Ile Asn Trp Lys Val Arg Lys Gln Leu Pro Val Ile Arg
1               5                   10                  15

Gln Thr Glu Ser Ala Glu Cys Gly Leu Ala Cys Leu Ala Met Ile Ala
            20                  25                  30

Cys Trp His Gly Leu Lys Thr Asp Leu Ser Thr Leu Arg Glu Arg Phe
        35                  40                  45

Asn Ile Gly Ile Gln Gly Met Thr Leu Gln Arg Leu Ile Glu Cys Ala
    50                  55                  60

Ala Ser Ile His Leu Ser Ser Arg Ala Val Arg Leu Glu Pro Glu Asp
65                  70                  75                  80

Leu Arg Cys Leu Asn Leu Pro Ser Ile Leu His Trp Asp Met Asn His
                85                  90                  95

Phe Val Val Leu His Lys Val Arg Gly Asn Arg Leu Tyr Ile His Asp
            100                 105                 110

Pro Asp Arg Gly Lys Ile Thr Ile Ser Leu Leu Asp Ala Gly Lys His
        115                 120                 125

Phe Thr Gly Val Ala Leu Glu Leu Thr Pro Ala Ser Asp Phe Thr Pro
    130                 135                 140
```

-continued

```
Arg Asn Glu Arg Lys Lys Ile His Leu Arg Gln Leu Thr Gly Lys Thr
145                 150                 155                 160

Pro Gly Leu Leu Ala Ser Met Thr Lys Ile Ile Ile Phe Ala Leu Ala
                165                 170                 175

Leu Glu Ile Leu Ala Leu Gly Gly Pro Leu Leu Asn Gln Leu Val Ile
            180                 185                 190

Asp Glu Val Leu Val Ala Ala Asp Arg Ser Leu Leu Tyr Val Ile Ile
        195                 200                 205

Val Ala Leu Leu Leu Leu Ser Leu Ile Gln Leu Leu Leu Ser Leu Ala
    210                 215                 220

Arg Gln Trp Ala Thr Ile Ser Leu Ser Val Asn Phe Asn Met Gln Trp
225                 230                 235                 240

Thr Ala Arg Val Phe His His Leu Val Arg Leu Pro Leu Ala Trp Phe
                245                 250                 255

Asp Ala Arg Ser Lys Gly Ser Ile Asn Ala Arg Phe Glu Ala Val Asp
            260                 265                 270

Ile Ile Gln Gln Ala Leu Thr Thr Gln Val Leu Glu Gly Ile Leu Asp
        275                 280                 285

Met Leu Leu Ile Val Thr Ala Leu Cys Met Met Leu Leu Tyr Ser Pro
    290                 295                 300

Gly Met Thr Leu Ile Ala Val Ile Ala Ala Ile Ile Tyr Gly Ala Leu
305                 310                 315                 320

Arg Ala Leu Trp Tyr Pro Ala Leu Arg Gln Ser Val Glu Asp Val Trp
                325                 330                 335

Asp Ala Gly Thr Lys Glu Ser Gly His Phe Leu Glu Thr Leu Asn Gly
            340                 345                 350

Ile Gln Ser Leu Arg Ile Asn Gly Val Thr Ile His Arg Glu Ala Ala
        355                 360                 365

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atcactataa atatgagttg gtgcgtttcg acccagtgag ttacgagccc cactggaacg   4680

gtcattacgg gggccacctt caaagttgct gtttgcgttt cctccgctga caagcgttat   4740

ctcatctaaa gttaattctc ttatatttgc cat                                 4773
```

<210> SEQ ID NO 35
<211> LENGTH: 102
<212> TYPE: PRT
<213> ORGANISM: Escherichia coli

<400> SEQUENCE: 35

```
Asn Ala Asn Ser Asn Phe Glu Gly Gly Pro Arg Asn Asp Arg Ser Ser
1               5                   10                  15

Gly Ala Arg Asn Ser Leu Gly Arg Asn Ala Pro Thr His Ile Tyr Ser
            20                  25                  30

Asp Pro Ser Thr Val Lys Cys Ala Asn Ala Val Phe Ser Gly Met Ile
        35                  40                  45

Gly Gly Ala Ile Lys Gly Gly Pro Ile Gly Met Ala Arg Gly Thr Ile
    50                  55                  60

Gly Gly Ala Val Val Gly Gln Cys Leu Ser Asp His Gly Ser Gly Asn
65                  70                  75                  80

Gly Ser Gly Asn Arg Gly Ser Ser Ser Ser Cys Ser Gly Asn Asn Val
                85                  90                  95

Gly Gly Thr Cys Asn Arg
            100
```

<210> SEQ ID NO 36
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 36

```
tgctgcgatg gaaaaacgtc                                                  20
```

<210> SEQ ID NO 37
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 37

```
ttctggacgc ttgcgatctt                                                  20
```

```
<210> SEQ ID NO 38
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 38

cttgcagacg tcttgcagtc ttaaggggac tggagc                               36


<210> SEQ ID NO 39
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 39

gccttgcggc ctgccctaag gcaagccgcc agacgt                               36


<210> SEQ ID NO 40
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 40

atcgctgtag gtctgggtct                                                 20


<210> SEQ ID NO 41
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 41

atgtcctgcc agcgttctac                                                 20


<210> SEQ ID NO 42
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 42

aatatcagaa cgttaactaa atagaggcat tgtgct                               36


<210> SEQ ID NO 43
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 43

ctaccgtaat aaattcagac atcagcccct ccctcc                               36


<210> SEQ ID NO 44
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
```

```
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 44

agcggcagga tgcattatca                                                  20


<210> SEQ ID NO 45
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 45

gggaagatta ctggctgcga                                                  20


<210> SEQ ID NO 46
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 46

gtgaatattc acgggcttta tgtaatttac attgaa                                36


<210> SEQ ID NO 47
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 47

aattaacacc tatgtattaa tcggagagag tagatc                                36


<210> SEQ ID NO 48
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 48

caatggcaga tgaagcgagc                                                  20


<210> SEQ ID NO 49
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 49

tgcaaatggg ctggatagca                                                  20


<210> SEQ ID NO 50
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 50

aactgcgcac tctatgcata ttgcagggaa atgatt                                36
```

```
<210> SEQ ID NO 51
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 51

caggaaaaaa gcgctcccgc aggagcgctg aaggga                              36


<210> SEQ ID NO 52
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 52

cgctatcagg gtaacgggag                                                20


<210> SEQ ID NO 53
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 53

agcactttca cggtagcgaa                                                20


<210> SEQ ID NO 54
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 54

gttgtcagaa tcgatctggt tgatgatgta gtcaac                              36


<210> SEQ ID NO 55
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 55

gtgatcgtcc ctgctctgtt agtagcaggt actgca                              36


<210> SEQ ID NO 56
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 56

tgttgcgaac ctttgggagt                                                20


<210> SEQ ID NO 57
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer
```

```
<400> SEQUENCE: 57

agcaaggtga cgatgagcaa                                                20


<210> SEQ ID NO 58
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 58

gggcaaatga acttcgtggc gagaagcgca atcgcc                              36


<210> SEQ ID NO 59
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer

<400> SEQUENCE: 59

cttacaaatt gttgcgaacc tttgggagta caaaca                              36


<210> SEQ ID NO 60
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer site

<400> SEQUENCE: 60

tgtgtaggct ggagctgctt cg                                             22


<210> SEQ ID NO 61
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide primer site

<400> SEQUENCE: 61

catatgaata tcctcctta                                                 19


<210> SEQ ID NO 62
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide

<400> SEQUENCE: 62

gaaaacatac aaattttttc acatatttac atttaatcac acaatttcac tttcattaca     60

tttttg                                                               66


<210> SEQ ID NO 63
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Sythetic oligonucleotide F1

<400> SEQUENCE: 63

tttacttttg gttacatatt                                                20
```

<210> SEQ ID NO 64
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide F2

<400> SEQUENCE: 64

ttttcttttt gaaaccaaat 20

<210> SEQ ID NO 65
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleutide F3

<400> SEQUENCE: 65

ttatctttgt agcactttca 20

<210> SEQ ID NO 66
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide F4

<400> SEQUENCE: 66

gttacggaat attacattgc 20

<210> SEQ ID NO 67
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide C1

<400> SEQUENCE: 67

tttacatttt gaaacatcta 20

<210> SEQ ID NO 68
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide B1

<400> SEQUENCE: 68

tttacattta atcacacaat 20

<210> SEQ ID NO 69
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide B2

<400> SEQUENCE: 69

ttcactttca ttacatttt 19

<210> SEQ ID NO 70
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic oligonucleotide B3

```
<400> SEQUENCE: 70

tacaaatttt ttcacata                                                  18


<210> SEQ ID NO 71
<211> LENGTH: 120
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic McsS

<400> SEQUENCE: 71

Met Ser Asn Ile Arg Glu Leu Ser Phe Asp Glu Ile Ala Leu Val Ser
1               5                   10                  15

Gly Gly Asn Ala Asn Ser Asn Tyr Glu Gly Gly Gly Ser Arg Ser Arg
            20                  25                  30

Asn Thr Gly Ala Arg Asn Ser Leu Gly Arg Asn Ala Pro Thr His Ile
        35                  40                  45

Tyr Ser Asp Pro Ser Thr Val Lys Cys Ala Asn Ala Val Phe Ser Gly
    50                  55                  60

Met Val Gly Gly Ala Ile Lys Gly Gly Pro Val Gly Met Thr Arg Gly
65                  70                  75                  80

Thr Ile Gly Gly Ala Val Ile Gly Gln Cys Leu Ser Gly Gly Gly Asn
                85                  90                  95

Gly Asn Gly Gly Gly Asn Arg Ala Gly Ser Ser Asn Cys Ser Gly Ser
            100                 105                 110

Asn Val Gly Gly Thr Cys Ser Arg
        115                 120


<210> SEQ ID NO 72
<211> LENGTH: 103
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic ColV

<400> SEQUENCE: 72

Met Arg Thr Leu Thr Leu Asn Glu Leu Asp Ser Val Ser Gly Gly Ala
1               5                   10                  15

Ser Gly Arg Asp Ile Ala Met Ala Ile Gly Thr Leu Ser Gly Gln Phe
            20                  25                  30

Val Ala Gly Gly Ile Gly Ala Ala Ala Gly Gly Val Ala Gly Gly Ala
        35                  40                  45

Ile Tyr Asp Tyr Ala Ser Thr His Lys Pro Asn Pro Ala Met Ser Pro
    50                  55                  60

Ser Gly Leu Gly Gly Thr Ile Lys Gln Lys Pro Glu Gly Ile Pro Ser
65                  70                  75                  80

Glu Ala Trp Asn Tyr Ala Ala Gly Arg Leu Cys Asn Trp Ser Pro Asn
                85                  90                  95

Asn Leu Ser Asp Val Cys Leu
            100


<210> SEQ ID NO 73
<211> LENGTH: 105
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic MccL

<400> SEQUENCE: 73
```

-continued

```
Met Arg Glu Ile Thr Leu Asn Glu Met Asn Asn Val Ser Gly Ala Gly
1               5                   10                  15

Asp Val Asn Trp Val Asp Val Gly Lys Thr Val Ala Thr Asn Gly Ala
            20                  25                  30

Gly Val Ile Gly Gly Ala Phe Gly Ala Gly Leu Cys Gly Pro Val Cys
        35                  40                  45

Ala Gly Ala Phe Ala Val Gly Ser Ser Ala Ala Val Ala Ala Leu Tyr
    50                  55                  60

Asp Ala Ala Gly Asn Ser Asn Ser Ala Lys Gln Lys Pro Glu Gly Leu
65                  70                  75                  80

Pro Pro Glu Ala Trp Asn Tyr Ala Glu Gly Arg Met Cys Asn Trp Ser
                85                  90                  95

Pro Asn Asn Leu Ser Asp Val Cys Leu
            100                 105


<210> SEQ ID NO 74
<211> LENGTH: 90
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Mcc24

<400> SEQUENCE: 74

Met Tyr Met Arg Glu Leu Asp Arg Glu Glu Leu Asn Cys Val Gly Gly
1               5                   10                  15

Ala Gly Asp Pro Leu Ala Asp Pro Asn Ser Gln Ile Val Arg Gln Ile
            20                  25                  30

Met Ser Asn Ala Ala Trp Gly Pro Pro Leu Val Pro Glu Arg Phe Arg
        35                  40                  45

Gly Met Ala Val Gly Ala Ala Gly Gly Val Thr Gln Thr Val Leu Gln
    50                  55                  60

Gly Ala Ala Ala His Met Pro Val Asn Val Pro Ile Pro Lys Val Pro
65                  70                  75                  80

Met Gly Pro Ser Trp Asn Gly Ser Lys Gly
                85                  90


<210> SEQ ID NO 75
<211> LENGTH: 99
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic MccE492

<400> SEQUENCE: 75

Met Arg Glu Ile Ser Gln Lys Asp Leu Asn Leu Ala Phe Gly Ala Gly
1               5                   10                  15

Glu Thr Asp Pro Asn Thr Gln Leu Leu Asn Asp Leu Gly Asn Asn Met
            20                  25                  30

Ala Trp Gly Ala Ala Leu Gly Ala Pro Gly Gly Leu Gly Ser Ala Ala
        35                  40                  45

Leu Gly Ala Ala Gly Gly Ala Leu Gln Thr Val Gly Gln Gly Leu Ile
    50                  55                  60

Asp His Gly Pro Val Asn Val Pro Ile Pro Val Leu Ile Gly Pro Ser
65                  70                  75                  80

Trp Asn Gly Ser Gly Ser Gly Tyr Asn Ser Ala Thr Ser Ser Ser Gly
                85                  90                  95

Ser Gly Ser
```

```
<210> SEQ ID NO 76
<211> LENGTH: 75
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic MchB

<400> SEQUENCE: 76

Met Arg Glu Ile Thr Glu Ser Gln Leu Arg Tyr Ile Ser Gly Ala Gly
1               5                   10                  15

Gly Ala Pro Ala Thr Ser Ala Asn Ala Ala Gly Ala Ala Ala Ile Val
            20                  25                  30

Gly Ala Leu Ala Gly Ile Pro Gly Gly Pro Leu Gly Val Val Val Gly
        35                  40                  45

Ala Val Ser Ala Gly Leu Thr Thr Ala Ile Gly Ser Thr Val Gly Ser
    50                  55                  60

Gly Ser Ala Ser Ser Ser Ala Gly Gly Gly Ser
65                  70                  75


<210> SEQ ID NO 77
<211> LENGTH: 84
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic cleaved mcpM

<400> SEQUENCE: 77

Arg Asn Ser Leu Gly Arg Asn Ala Pro Thr His Ile Tyr Ser Asp Pro
1               5                   10                  15

Ser Thr Val Lys Cys Ala Asn Ala Val Phe Ser Gly Met Ile Gly Gly
            20                  25                  30

Ala Ile Lys Gly Gly Pro Ile Gly Met Ala Arg Gly Thr Ile Gly Gly
        35                  40                  45

Ala Val Val Gly Gln Cys Leu Ser Asp His Gly Ser Gly Asn Gly Ser
    50                  55                  60

Gly Asn Arg Gly Ser Ser Ser Ser Cys Ser Gly Asn Asn Val Gly Gly
65                  70                  75                  80

Thr Cys Asn Arg
```

We claim:

1. A composition comprising

microcin MccPDI having one or more of a sequence of SEQ ID NO: 77 or a functional variant thereof and/or SEQ ID NO:24 or a functional variant thereof, wherein said functional variant has a sequence at least 95% identical to SEQ ID NO: 77 or SEQ ID NO:24; and an oxidizing agent.

2. The composition of claim **1**, wherein said composition further comprises at least 10% oxidizing agent.

3. The composition of claim **1**, wherein said composition further comprises at least 20% oxidizing agent.

4. The composition of claim **1**, wherein said composition further comprises dimethyl sulfoxide (DMSO).

* * * * *